REMARKS/ARGUMENTS

I. Status of the claims

Claims 1-5, 8, 10, 12-16, 21, 27, 21, 33-37, and 69-89 are pending. Claims 41-50 have been withdrawn from consideration as drawn to a non-elected invention. Claims 6, 7, 9, 11, 17-20, 22-26, 28-30, 32, 38-40, and 51-68 have been canceled herein and new claims 69-89 added.

II. The Amendments Herein

No new matter has been added by the present amendments.

Claims 1 and 27 have been amended to recite that the claimed polypeptides are isolated and have been amended to recite that the affinity for the antigen is relative to the affinity of the polypeptide for the same antigen. Claim 14 and the other claims that recite "*Pseudomonas* exotoxin" have been clarified to recite that the exotoxin referred to is *Pseudomonas* exotoxin A, as supported by, for example, page 37, lines 3-19, and to provide antecedence for the abbreviation "PE" used in the dependent claims. The claims that refer to PE have also been amended to delete the recitation "or a cytotoxic fragment thereof" since the term PE as defined in the specification includes truncated forms of the native protein. See, page 36, lines 26-31. Claims 33, 38, and 64 have been amended to recite "immunoconjugate" rather than "immunotoxin." Immunoconjugates are supported throughout the specification, including page 31, line 22, to page 32, line 9.

The new claims likewise do not add new matter. The new claims are drawn to antibodies that comprise the complementarity determining regions ("CDRs") as shown in Figure 1 with specific mutations of CDR3 of the V_H or V_L taught in the specification and, optionally, mutations in residues of CDRs 1 or 2 of the V_H or V_L encoded by codons which have a nucleotide in a hot spot motif. The CDR3 mutations are supported throughout the specification, including Figure 3. That CDRs other than CDR3 can be mutated in the same way as the CDR3s is supported throughout the specification, including page 23, lines 14-21. The various

embodiments claimed (antibodies, chimeric molecules, methods of killing cells bearing mesothelin, etc.) track those in the claims as originally presented.

The specification points out that light and heavy variable chains contain a framework region interrupted by the CDRs (see pages 12-13, bridging paragraph), and that the extent of the framework region and CDRs have been defined. *Id.* The specification also cites and incorporates the Kabat database, which aligns the CDRs of thousands of antibodies (the Kabat database is discussed at some length below in connection with one of the rejections), *id.*, and notes that the framework serves to position and align the CDRs in three dimensional space. The specification further states that the CDRs are primarily responsible for binding to an epitope of an antigen. Specification, at page 13, lines 9-10.

Due to the heightened bar for introducing evidence following issuance of a final office action, Applicants wish to introduce the following evidence at this time. As pointed out in the Applicants' response dated January 12, 2005, at page 13, it was known in the art for more than a decade before the priority date that antigen specificity can be changed by substituting the CDRs of one antibody for another. For example, the complementarity determining regions (CDRs) of an antibody from a non-human animal, such as a mouse, can be grafted onto a human antibody framework of known three dimensional structure to provide antibodies that bind with the specificity of the non-human antibody (see, e.g., the Jones, et al. Nature 321:522 (1986) reference whose abstract was provided with the January 2005 response. In addition, see, WO 87/02671; U.S. Patent Nos. 5,859,205 and 5,585,089; EP Patent Application 0173494; Verhoeyen, et al., Science 239:1534 (1988) (abstract enclosed), Riechmann, et al. Nature 332:323 (1988) (abstract enclosed); and Winter & Milstein, Nature 349:293 (1991)).

It has further been known in the art for years prior to the priority date that antibodies from mouse or other species can be "humanized" to reduce the immune response to the antibodies by replacing residues at various positions in the framework region with residues more commonly found at those positions in the framework region of human antibodies. See, e.g., U.S. Patent Nos. 6,180,377; 5,693,762; 5,585,089; and 5,530,101. All of the references cited above were available and known in the art and did not have to be taught in the present specification. The well established rule is that "the specification not need teach, and preferably

omits, that which is well known in the art." *See, e.g., In re Buchner*, 18 USPQ2nd 1331, 1332 (Fed. Cir. 1991).

In short, it was known in the art before the priority date that the CDRs determine antigen specificity and that they can be moved from one framework region to another. It was further known that the framework region of antibodies of one species can be modified by, for example, "humanization" without losing antigen specificity. The new claims are drawn to antimesothelin antibodies that comprise the CDRs set forth in Figure 1, with specific substitutions set forth in Figure 3 and, optionally, like substitutions in the other CDRs. They are therefore supported by both the specification and by the knowledge common in the art as of the priority date. No new matter has been added.

III. The Office Action

Applicants note with appreciation that the current Action withdraws a number of grounds of rejection made in the previous Office Action.

The current Action rejects claims 1-40 and 51-66 on a variety of grounds. Applicants amend in part and traverse all the rejections. The rejections are discussed separately below in the order in which they appear in the Action.

A. Objections for Informalities

1. Claim 7 is objected to for an unnecessary space in the word "substitutions." Claim 7 has been canceled.

2. Claim 63 is objected to as a substantial duplicate of claim 31, should that claim be found allowable. Claim 63 has been canceled.

B. Rejection under 35 U.S.C. § 101

Claims 1-6, 27, 28, 51-54, 60 and 61 are rejected under 35 U.S.C. § 101 as directed to non-statutory subject matter. According to the Action, the claims do not sufficiently distinguish the claimed polypeptides and other embodiments from those that might occur

naturally. The claims have been amended to recite "isolated" as helpfully suggested by the Examiner.

C. Rejections under 35 U.S.C. § 112, second paragraph

1. Rejection for lack of clarity as to binding affinity

Claims 1-21, 27-29, 31-40 and 51-59 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite for reciting "having at least 5 times higher binding affinity." According to the Action, it is unclear to what the higher affinity is being compared. The Action states that it is unclear if the claimed polypeptides displace the parental antibody or if the parental antibody is bound to the same or a different epitope at the same time as the claimed polypeptides, and suggests the use of the phrase "relative to the affinity of the parental antibody." The claims have been amended to clarify that the measurement is relative to the affinity of the parental antibody for the same antigen.

2. Rejection for lack of clarity as to whether cells contain antigen bound by the immunotoxin

Claims 33-37 are rejected under §112 because the claims recite a method of killing cells bearing an antigen but do not specify that the antigen bound by the immunotoxin is the same as the one expressed on the target cells. The claims have been amended to recite that the antigen bound by the immunotoxin is present on the cells to be killed by the claimed methods.

3. Rejection for phrase "a nucleic acid molecule."

Claims 31, 32, and 63 are rejected for lack of appropriate basis for the phrase " a nucleic acid molecule." Claim 31 has been amended to provide appropriate basis, and claims 32 and 63 have been canceled.

4. Rejection for recitation of antibodies by laboratory designations

Claims 6, 9, 11, 17-20, 22-26, 28-30, 32, 35-40, 51-62 and 64-66 are rejected as indefinite for recitation of "SS antibody" and "antibody E4" as the sole means of identifying the antibody. According to the Action, the use of laboratory designations renders claims indefinite because different laboratories could use the same designations. The Action suggests that this rejection can be obviated by amending the claims to provide unique identifiers for the antibodies.

The claims reciting the antibodies called SS1 and E4 have been canceled and rewritten as new claims 69-89. The new claims recite the amino acid substitutions in the CDRs that differentiate the antibodies from the parental antibody, called "SS."

5. Rejection for alleged inaccurate numbering of the SS antibody residues

Claims 6, 7, 9, 11, 17-20, 22-26, 28-30, 32, 35-40, 54, 55, 61, 62, and 65 are rejected as indefinite because the claims recite amino acid substitutions at specific positions in antibody SS. This rejection has several parts, which for clarity are addressed individually.

a. Allegation that the complete sequence of antibody SS is not shown

The Action alleges that the claims do not define the complete sequence of the SS antibody. Applicants respectfully note that the claims already did define the complete sequence, but this may not have been fully appreciated as will become clearer in the next section. The new claims recite the amino acid substitutions in the CDRs that differentiate the antibodies from the parental SS antibody.

b. Allegation that the antibody represented by SEQ ID NO:1 does not contain the specific amino acid residues at the positions recited in the claims

The Action next states that the antibody represented by SEQ ID NO:1 does not contain the specific amino acid residues at the positions recited in the claims. Action, at pages 7-8, bridging lines. It then notes that, if one counts sequentially from the amino terminus of the heavy and light chains, the residues do not seem to correspond to those identified in the claims by their position numbers.

Applicants respectfully note that the residues of antibodies are typically numbered according to one of two numbering systems which align the sequences so that the complementarity determining regions can be determined. The Kabat system has been used in the art for over thirty years and is the most widely used. For the Examiner's information, an article by the persons maintaining the Kabat database in the year 2000 and looking back over the first 30 years of its use in the art is attached. See, Johnson and Wu, "Kabat Database and its applications: 30 years after the first variability plot," Nucleic Acids Res. 28(1):214-218 (2000). The present application uses the 1991 edition of the Kabat numbering system, which is incorporated by reference at page 13, lines 1-4 of the specification. Persons of skill would understand that the numbering is according to the Kabat system both by their knowledge of the art, the reference to the Kabat database in the Definitions section, and even more specifically by the fact that the Kabat subgroup and family of each chain of the SS antibody is identified in the specification at page 23, lines 26-29.

As noted, numbering under the Kabat system is meant to align the CDRs. Persons of skill are aware that, under the Kabat system, residues in the CDRs are frequently assigned the same number, but different letters (e.g., 27A-G) to maintain the alignments. Therefore, numbering under the Kabat system can and usually does differ from simply counting the residues from the amino terminus end.

For example, in Schier et al., J Mol Biol. 263:551-567 (1996), which is already in the record as one of the references cited by the Action against the application with respect to the obviousness and double patenting rejections, Table 2 on page 554 notes that the numbering is according to Kabat, and numbers the 11 residues of the V_L CDR3 sequentially from 89 through 97, including a residue numbered 95, one numbered as residue 95a, and one numbered as residue 95b. Merely sequentially numbering the 11 residues, the residues would be numbered residues 89 to 99. Similarly, the text on the bottom left of the same page refers to residues 95 to 97, 100a to 100d, and 100g to 102 being separately mutated. At page 556, Table 3 shows the effect of mutating various residues of the V_H CDR3 of antibody C6.5 to alanine. The residues listed include residues 100, 100a, 100b, 100c, 100d, 100g, 100h, 100i, 100j, 100k, and 100l.

Clearly, simply counting sequentially from the amino terminus of the V_L or of the V_H chains of antibody C6.5 would give different numbers to the residues than using the Kabat system. The same is true, though less dramatically, with respect to the SS antibody which is the subject of SEQ ID NO:1. While the above discussion is sufficient to explain the difference between the numbering underlying the rejection and that used in the application, for extra measure, the face page and two pages of U.S. Patent No. 6,875,433 are attached. The pages of the '433 patent provide more background information, in textual form, of the understanding in the art of the Kabat system's method of aligning sequences and of numbering residues.

Since the Kabat system has been known and used in the art since 1970 (as noted in the Johnson and Wu publication, *supra*), persons in the art are well familiar with determining position numbers under the system. Nonetheless, to reduce any possible ambiguity, the claims reciting the various anti-mesothelin antibodies have been cancelled and rewritten as new claims which recite substitutions in residues that are numbered as shown in Figure 3.

In short, the claims as presented were clear to persons of skill in the art, who had close to thirty years of familiarity with the Kabat numbering system prior to the priority date of the present application. For extra measure, the new claims recite that the residues are numbered as shown in a figure of the application. Accordingly, the claims as presented are not indefinite.

c. Allegation that the laboratory designations of antibodies render the claims indefinite

The Action further notes that the claims recite laboratory designations, SS1, D8 and C10 in parentheses, and alleges that this renders the claim indefinite because different laboratories may use the same designation to define different molecules. Action, at page 8. Applicants amend in part and traverse.

The claims that mentioned the names of the various antibodies in parentheses already also recited the particular mutations contained compared to the SS antibody. The claims in question have been canceled and rewritten to recite specific mutations. There is thus no possibility that the laboratory designations of the antibodies will cause confusion with designations of other antibodies. On the other hand, leaving the names of the antibodies in the

parentheses aids the reader. Figure 4, for example, refers to the antibodies by their laboratory designations. Retaining the terms in the parentheses therefore enhances the ability of the reader to readily compare the characteristics of the claimed antibodies as set forth in the specification.

Reconsideration and withdrawal of the rejection is respectfully requested.

D. Rejection under 35 U.S.C. § 103(a) over Chowdhury in view of Wagner, Schier, and Pastan

Claims 1-6, 8-21, 27, 28, 31-34, 36, 37, 51-54, 56-61, 63, 64, and 66 are rejected under 35 U.S.C. § 103(a) as obvious over Chowdhury et al., Proc Natl Acad Sci USA 95:669-674 (1998) ("Chowdhury") in view of Wagner et al., Nature 376:732 (1995) ("Wagner"), Pastan et al., U.S. Patent No. 6,083,502 ("Pastan," or the "'502 patent"), and Schier et al., J Mol Biol 263:551-567 (1996) ("Schier"). Action, at pages 8-13. The Action reiterates that Chowdhury teaches the high affinity anti-mesothelin antibody SS. It further reiterates that Wagner teaches that hot spot motifs are preferred targets for mutation and that most hot spots are associated with AGY serine codons. Action, at page 10. It further states that Scheir teaches the restriction of mutagenesis to the CDRs located in the middle of the binding site of an scFv that targets c-erbB-2 produces scFv affinities in the low nanomolar range. The Pastan '502 patent is stated to teach the mesothelin antigen and the K1 antibody and methods of using anti-mesothelin antibodies to target cytotoxins to mesothelin-expressing cells.

Applicants have previously pointed out that the SS antibody taught by Chowdhury was stated to be a high affinity antibody which had been selected for by multiple rounds of phage display, that Wagner's teaching regard the normal maturation of antibody affinity, and that Wagner contains no teaching or suggestion that antibodies which have been selected for high affinity through the artificial process of phage display can undergo further increases in affinity through mutations in CDR hotspots. Therefore, Applicants maintained that the person of skill would be likely to conclude that the antibodies selected by the phage display process, and displaying the affinity reported in Chowdhury had already undergone mutation at these hot spots and obtained whatever benefit was available from such mutations. Applicants further observed that Wagner contains no teaching or suggestion that the process of affinity

maturation it describes for natural antibodies would permit generation of antibodies with 5 times the affinity of a parental antibody, and therefore does not teach or suggest all of the recitations of the claims under examination.

In the prior Action, these references were combined with Adams et al., Cancer Research 58:485-490 (1998) ("Adams"). Applicants pointed out that Adams did not make up the deficiency in the Chowdhury and Wagner references, since Adams was only cited to indicate that higher affinity antibodies are generally desirable as targeting agents. Therefore, the Applicants maintained that the combination of references made by the Action did not teach or suggest the invention as claimed.

The current Action tries to make up for the deficiency of the previous obviousness rejection by adding Schier, which teaches the improvement of an antibody known as C6.5. The Action argues that Schier teaches the restriction of mutagenesis to the CDRs located in the middle of the binding site of an scFv that targets c-erbB-2 produces scFv affinities in the low nanomolar range.

The authors of Schier were themselves aware of the teachings of Wagner. The Schier authors in fact cite Wagner in discussing the observation that nucleotide substitutions in vivo occur at mutational hotspots, and in particular at serine residues encoded by AGY rather than TCN. See, Schier, at page 561, left column. They further mention that three serines in the sequence motif CSSNC in the V_H CDR3 of the C6.5 antibody were encoded by AGY and were hotspots for substitutions which increased affinity. *Id.* And, the authors of Schier were also, of course, very familiar with their own work. But, after considering at some length both the Wagner teachings and their own finding in their Discussion section, the Schier authors set forth their conclusions on a "mutagenesis strategy for efficiently increasing antibody fragment affinity", page 562 at left column, bottom paragraph, - and it does not include focusing mutations on hot spot motifs.

What the Schier authors indicate that Wagner and their own work leads to as a strategy is that "[m]utagenesis should be directed into V_L and V_H CDR3 sequentially, as in this work" that "[m]odeling should be used to identify CDR residues that are likely to have a structural role," as "[t]hese residues are conserved, leaving at most four to five residues to be

completely randomized." *Id.* With respect to the V_H, they suggest randomizing four to five residues at a time. *Id.* Thus, despite their knowledge of their own findings, and their knowledge of Wagner, the Schier authors do not teach restricting mutations to the residues encoded by RGYW or AGY hot spots, but rather to randomizing at one time four or five residues in a CDR that are not identified as having a structural role. Indeed, the RGYW hot spot motif does not appear to be mentioned at all in Schier.

The Examiner is respectfully reminded that the teachings of a prior art must be considered "as a whole." See, MPEP 2141.02. Given the large increases in affinity achieved by the Schier authors, and the Schier authors' careful consideration and discussion of Wagner, persons of skill in the art taking the teachings of Wagner and Schier as a whole would have followed the teachings of Schier to achieve large increases in the affinity of antibodies. And, as shown above, those teachings do not rely on mutating only the residues encoded by nucleotides in hot spot motifs. Instead, Schier teaches identifying non-structural residues and then mutating four to five of these non-structural residues at a time, sequentially first in the V_L CDR3 and then in the V_H CDR3.

Looked at in another way, Schier showed a solution for the problem of creating large increases in affinity over a starting parental antibody, and did so with a detailed knowledge and careful consideration of the Wagner reference. The Schier authors did not see their own results as suggesting that mutations should be directed to residues encoded by nucleotides in hot spot motifs. It would therefore seem apparent that the Action's conclusion that the combined teachings of Schier and Wagner suggest mutating only those residues is a hindsight recreation of the invention driven by a knowledge of the present disclosure.

The conclusion that the work embodied in the present disclosure would have been obvious at the time the invention was made is further rebutted by the fact that sophisticated persons of skill in the art at the time the invention was made did not consider it obvious, as shown by the fact that they considered it worthy of publication in one of the most prestigious and highest impact scientific journals. The work disclosed in the present specification was in fact published (following the priority date) in Nature Biotechnology. See, Chowdhury and Pastan, Nature Biotechnology 17:568-572 (1999) (a copy of this publication is enclosed for the

Examiner's convenience). The editors of Nature Biotechnology can be considered both independent and knowledgeable in the state of the relevant art at the time of publication. If those editors had considered the present invention obvious in light of the teachings in the art at the time, it is unlikely they would accepted the paper for publication in their journal. It is understood that this is only a secondary consideration of non-obviousness and that journal editors do not make their selections based on the criteria of §103 (a). Nonetheless, the publication in a major, high impact scientific journal reflects the contemporaneous judgment of unbiased persons of skill at the time that the work embodied in the publication was significant and worthy of wide dissemination in the scientific community.

The Pastan '502 patent is stated as teaching the mesothelin antigen and methods for targeting cells bearing mesothelin, and as teaching the K1 antibody and using anti-mesothelin antibodies to target cytotoxins to target cells. Therefore, nothing in the Pastan '502 patent help support the rejection's contention that it would have been obvious to target mutations of an antibody to CDR residues encoded by a hot spot motif. That argument has to stand or fall on what would have been obvious in view of Wagner and Schier. As noted above, Wagner and Schier neither teach nor suggest the invention as claimed.

Applicants respectfully request reconsideration and withdrawal of the rejection in view of the discussion above.

E. Rejection for Obviousness-type double patenting over U.S. Patent No. 6,809,184 in view of Chowdhury, Wagner, Schier, and Pastan

Claims 1-7, 8-21, 51-54, 56-59 are rejected under the judicially created doctrine of obviousness-type double patenting over U.S. Patent No. 6,809,184 (the "184 patent") in view of Chowdhury, Wagner, the Pastan '502 patent, and Schier. Action, at pages 14-17. Applicants traverse. According to the Action, the '184 patent sets forth the SS antibody, but doesn't teach mutating only CDR residues encoded by codons having nucleotides in hot spot motifs. The Action argues that the claims in the current application are obvious variants of the claims of the '184 patent because it would have been prima facie obvious to one of ordinary skill in the art to have only mutated CDR hot spots in the SS antibody. Applicants traverse the rejection.

The only element in the double patenting rejection not present in the obviousness rejection discussed in the preceding section is the addition of the '184 patent, which teaches the SS antibody already taught by the Chowdhury reference, which was the report in the scientific literature of the work embodied in the '184 patent. Thus, the contention that there is obviousness-type double patenting necessarily rests, as did the obviousness rejection, on the alleged obviousness of mutating only residues encoded by nucleotides in hot spot motifs in view of Wagner and Schier. This contention is false, as shown in the preceding section. For completeness of the response, the discussion below restates the reasons the Action's combination of Wagner and Schier does not render obvious the present invention.

First, as noted in the preceding section, persons of skill at the time the invention was made did not consider it obvious; to the contrary, they considered it worthy of publication in one of the most prestigious and high impact scientific journals. The work disclosed in the present specification was published (after the priority date) in Nature Biotechnology. See, Chowdhury and Pastan, Nature Biotechnology 17:568-572 (1999) (copy enclosed). The editors of Nature Biotechnology can be considered both independent and knowledgeable in the state of the relevant art at the time of publication. If those editors had considered the present invention obvious in light of the teachings in the art at the time, it is unlikely they would accepted the paper for publication in their journal. Thus, the contemporaneous judgment of unbiased persons of skill at the time reflects that they deemed the invention significant and worthy of wide dissemination in the scientific community. While editors of scientific journals do not make their decisions based on the criteria of §103(a), their judgment can be considered at least as a secondary consideration of non-obviousness which requires reconsideration and reweighting of the rejection.

Second, the rejection relies on the same combination of Chowdhury, Wagner, Schier and the Pastan '502 patent discussed in the previous section. Applicants pointed out in a previous response that the SS antibody taught by Chowdhury was stated to be a high affinity antibody which had been selected for by multiple rounds of phage display, that Wagner's teaching regard the normal maturation of antibody affinity, and that Wagner contains no teaching or suggestion that antibodies which have been selected for high affinity through the artificial

process of phage display can undergo further increases in affinity through mutations in CDR hotspots. Therefore, Applicants maintained that the person of skill would be likely to conclude that the antibodies selected by the phage display process, and displaying the affinity reported in Chowdhury had already undergone mutation at these hot spots and obtained whatever benefit was available from such mutations. Applicants further observed that Wagner contains no teaching or suggestion that the process of affinity maturation it describes for natural antibodies would permit generation of antibodies with 5 times the affinity of a parental antibody, and therefore does not teach or suggest all of the recitations of the claims under examination.

In the prior Action, these references were combined with Adams et al., Cancer Research 58:485-490 (1998) ("Adams"). Applicants pointed out that Adams did not make up the deficiency in the Chowdhury and Wagner references, since Adams was only cited to indicate that higher affinity antibodies are generally desirable as targeting agents. Therefore, the Applicants maintained that the combination of references made by the Action did not teach or suggest the invention as claimed.

The current Action tries to make up for the deficiency of the previous obviousness rejection by adding Schier, which teaches the improvement of an antibody known as C6.5. The Action argues that Schier teaches the restriction of mutagenesis to the CDRs located in the middle of the binding site of an scFv that targets c-erbB-2 produces scFv affinities in the low nanomolar range.

The authors of Schier were themselves aware of the teachings of Wagner. The Schier authors in fact cite Wagner in discussing the observation that nucleotide substitutions in vivo occur at mutational hotspots, and in particular at serine residues encoded by AGY rather than TCN. See, Schier, at page 561, left column. They further mention that three serines in the sequence motif CSSNC in the V_H CDR3 of the C6.5 antibody were encoded by AGY and were hotspots for substitutions which increased affinity. *Id.* And, the authors of Schier were also, of course, very familiar with their own work. But, after considering at some length both the Wagner teachings and their own finding in their Discussion section, the Schier authors set forth their conclusions on a "mutagenesis strategy for efficiently increasing antibody fragment

affinity", page 562 at left column, bottom paragraph, - and it does not include focusing mutations on hot spot motifs.

What the Schier authors indicate that Wagner and their own work leads to as a strategy is that "[m]utagenesis should be directed into V_L and V_H CDR3 sequentially, as in this work" that "[m]odeling should be used to identify CDR residues that are likely to have a structural role," as "[t]hese residues are conserved, leaving at most four to five residues to be completely randomized." *Id.* With respect to the V_H, they suggest randomizing four to five residues at a time. *Id.* Thus, despite their knowledge of their own findings, and their knowledge of Wagner, the Schier authors do not teach restricting mutations to the residues encoded by RGYW or AGY hot spots, but rather to randomizing at one time four or five residues in a CDR that are not identified as having a structural role. Indeed, the RGYW hot spot motif does not appear to be mentioned at all in Schier.

The Examiner is respectfully reminded that the teachings of a prior art must be considered "as a whole." See, MPEP 2141.02. Given the large increases in affinity achieved by the Schier authors, and the Schier authors' careful consideration and discussion of Wagner, persons of skill in the art taking the teachings of Wagner and Schier as a whole would have followed the teachings of Schier to achieve large increases in the affinity of antibodies. And, as shown above, those teachings do not rely on mutating only the residues encoded by nucleotides in hot spot motifs. Instead, Schier teaches identifying non-structural residues and then mutating four to five of these non-structural residues at a time, sequentially first in the V_L CDR3 and then in the V_H CDR3.

Looked at in another way, Schier showed a solution for the problem of creating large increases in affinity over a starting parental antibody, and did so with a detailed knowledge and careful consideration of the Wagner reference. The Schier authors did not see their own results as suggesting that mutations should be directed to residues encoded by nucleotides in hot spot motifs. It would therefore seem apparent that the Action's conclusion that the combined teachings of Schier and Wagner suggest mutating only those residues is a hindsight recreation of the invention driven by a knowledge of the present disclosure.

Applicants respectfully request reconsideration and withdrawal of the rejection in view of the remarks herein.

F. Rejection for Obviousness-type double patenting over U.S. Patent No. 6,083,502 in view of Chowdhury, Wagner, Schier, and Queen

Claims 1-6, 8-21, 33, 34, 51-54, 56-59, 64, and 66 are rejected under the judicially created doctrine of obviousness-type double patenting over the Pastan '502 patent in view of Chowdhury, Wagner, Schier, and Queen, U.S. Patent No. 5,530,101 ("Queen"). Action, at pages 17-22. Applicants traverse.

This second obviousness-type double patenting rejection differs from the one discussed in the preceding section in two respects. First, the rejection adds the Queen patent, which is cited as teaching monoclonal antibodies which can be conjugated to various cytotoxic agents. Second, the rejection adds the Examiner's concern that the current application and the '502 patent were not commonly owned at the time the invention in this application was made and indicates that the assignee can show, under 37 C.F.R. § 1.78(c), that the conflicting inventions were commonly owned at the time the invention was made.

To address the common ownership concern first, Applicants note that §1.78(c) addresses "an application or a patent under reexamination and at least one other application naming different inventors." The present rejection concerns an application and a patent that is not under reexamination, and, therefore, 37 C.F.R. §1.78 (c) is not applicable on it face to the present application. For the sake of good order, however, the undersigned counsel for the assignee states that the present application and the '502 patent have at all times been owned by the same assignee, the Government of the United States as represented by the Secretary of Health and Human Services.

Turning back to the substance of the is obviousness-type double patenting rejection, it rests, as does the double obviousness rejection discussed in the preceding section, on the alleged obviousness of mutating only residues encoded by nucleotides in hot spot motifs in view of Wagner and Schier. This contention is false, as shown in the preceding section. For

completeness of the response, the discussion below restates the reasons the Action's combination of Wagner and Schier does not render obvious the present invention.Before turning to the

First, as noted in the preceding section, persons of skill at the time the invention was made did not consider it obvious; to the contrary, they considered it worthy of publication in one of the most prestigious and high impact scientific journals. The work disclosed in the present specification was published (after the priority date) in Nature Biotechnology. See, Chowdhury and Pastan, Nature Biotechnology 17:568-572 (1999) (copy enclosed). The editors of Nature Biotechnology can be considered both independent and knowledgeable in the state of the relevant art at the time of publication. If those editors had considered the present invention obvious in light of the teachings in the art at the time, it is unlikely they would accepted the paper for publication in their journal. Thus, the contemporaneous judgment of unbiased persons of skill at the time reflects that they deemed the invention significant and worthy of wide dissemination in the scientific community. While editors of scientific journals do not make their decisions based on the criteria of §103(a), their judgment can be considered at least as a secondary consideration of non-obviousness which requires reconsideration and reweighting of the rejection.

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would permit generation of antibodies with 5 times the affinity of a parental antibody, and therefore does not teach or suggest all of the recitations of the claims under examination.

In the prior Action, these references were combined with Adams et al., Cancer Research 58:485-490 (1998) ("Adams"). Applicants pointed out that Adams did not make up the deficiency in the Chowdhury and Wagner references, since Adams was only cited to indicate that higher affinity antibodies are generally desirable as targeting agents. Therefore, the Applicants maintained that the combination of references made by the Action did not teach or suggest the invention as claimed.

The current Action tries to make up for the deficiency of the previous obviousness rejection by adding Schier, which teaches the improvement of an antibody known as C6.5. The Action argues that Schier teaches the restriction of mutagenesis to the CDRs located in the middle of the binding site of an scFv that targets c-erbB-2 produces scFv affinities in the low nanomolar range.

The authors of Schier were themselves aware of the teachings of Wagner. The Schier authors in fact cite Wagner in discussing the observation that nucleotide substitutions in vivo occur at mutational hotspots, and in particular at serine residues encoded by AGY rather than TCN. See, Schier, at page 561, left column. They further mention that three serines in the sequence motif CSSNC in the V_H CDR3 of the C6.5 antibody were encoded by AGY and were hotspots for substitutions which increased affinity. *Id.* And, the authors of Schier were also, of course, very familiar with their own work. But, after considering at some length both the Wagner teachings and their own finding in their Discussion section, the Schier authors set forth their conclusions on a "mutagenesis strategy for efficiently increasing antibody fragment affinity", page 562 at left column, bottom paragraph, - and it does not include focusing mutations on hot spot motifs.

What the Schier authors indicate that Wagner and their own work leads to as a strategy is that "[m]utagenesis should be directed into V_L and V_H CDR3 sequentially, as in this work" that "[m]odeling should be used to identify CDR residues that are likely to have a structural role," as "[t]hese residues are conserved, leaving at most four to five residues to be completely randomized." *Id.* With respect to the V_H, they suggest randomizing four to five

residues at a time. *Id.* Thus, despite their knowledge of their own findings, and their knowledge of Wagner, the Schier authors do not teach restricting mutations to the residues encoded by RGYW or AGY hot spots, but rather to randomizing at one time four or five residues in a CDR that are not identified as having a structural role. Indeed, the RGYW hot spot motif does not appear to be mentioned at all in Schier.

The Examiner is respectfully reminded that the teachings of a prior art must be considered "as a whole." See, MPEP 2141.02. Given the large increases in affinity achieved by the Schier authors, and the Schier authors' careful consideration and discussion of Wagner, persons of skill in the art taking the teachings of Wagner and Schier as a whole would have followed the teachings of Schier to achieve large increases in the affinity of antibodies. And, as shown above, those teachings do not rely on mutating only the residues encoded by nucleotides in hot spot motifs. Instead, Schier teaches identifying non-structural residues and then mutating four to five of these non-structural residues at a time, sequentially first in the V_L CDR3 and then in the V_H CDR3.

Looked at in another way, Schier showed a solution for the problem of creating large increases in affinity over a starting parental antibody, and did so with a detailed knowledge and careful consideration of the Wagner reference. The Schier authors did not see their own results as suggesting that mutations should be directed to residues encoded by nucleotides in hot spot motifs. It would therefore seem apparent that the Action's conclusion that the combined teachings of Schier and Wagner suggest mutating only those residues is a hindsight recreation of the invention driven by a knowledge of the present disclosure.

Applicants respectfully request reconsideration and withdrawal of the rejection in view of the remarks herein.

IV. Supplemental IDS

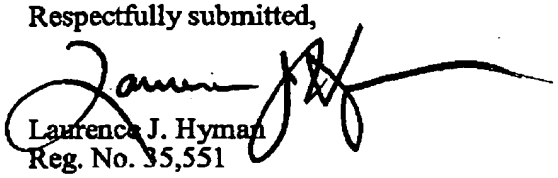
A supplemental information disclosure statement (IDS) is being filed under separate cover. The Examiner is respectfully requested to consider the references set forth in the Supplemental IDS.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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Kabat Database and its applications: 30 years after the first variability plot

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ABSTRACT

The Kabat Database was initially started in 1970 to determine the combining site of antibodies based on the available amino acid sequences at that time. Bence Jones proteins, mostly from human, were aligned, using the now-known Kabat numbering system, and a quantitative measure, variability, was calculated for every position. Three peaks, at positions 24–34, 50–56 and 89–97, were identified and proposed to form the complementarity determining regions (CDR) of light chains. Subsequently, antibody heavy chain amino acid sequences were also aligned using a different numbering system, since the locations of their CDRs (31–35B, 50–65 and 95–102) are different from those of the light chains. CDRL1 starts right after the first invariant Cys 23 of light chains, while CDRH1 is eight amino acid residues away from the first invariant Cys 22 of heavy chains. During the past 30 years, the Kabat database has grown to include nucleotide sequences, sequences of T cell receptors for antigens (TCR), major histocompatibility complex (MHC) class I and II molecules and other proteins of immunological interest. It has been used extensively by immunologists to derive useful structural and functional information from the primary sequences of these proteins. An overall view of the Kabat Database and its various applications are summarized here. The Kabat Database is freely available at http://immuno.bme.nwu.edu

INTRODUCTION

The purpose of maintaining the Kabat Database of aligned sequences of proteins of immunological interest, in our opinion, is to provide useful correlations between structure and function for this special group of proteins from their nucleotide and amino acid sequences to their tertiary structures (1). These sequences are thus aligned with the ultimate aim of understanding how these proteins are folded and how they can perform their biological functions. We include only coding region sequences that have been published. In some cases, only the amino acid sequences were published, while the corresponding nucleotide sequences were deposited in GenBank. All stored sequences were then printed out and checked visually against available published sequences. We routinely survey for possible new sequences in journals in our libraries, Medline entries, cross-references from other papers, and author notification; however, we may still miss some sequences. GenBank, on the other hand, contains a substantial number of unpublished sequences. If there are doubts about these sequences or their annotations, please refer to the original papers. The Kabat numbering systems (see the Introduction of 2) for antibody light and heavy chains, for TCR alpha and beta chains, etc., go hand-in-hand with variability calculations. The locations of the CDRs are the theoretically derived positions which can be verified experimentally. Indeed, from the first antigen–antibody Fab complex (3) to the complexes of TCR, processed peptide and MHC class I molecule (4,5), it has been realized that alignment of amino acid sequences and variability calculations can be of utmost importance in understanding how these important macromolecules function biologically. Due to the rapid development of genetic and protein engineering methods, mouse and rat antibodies have been humanized to treat human cancers, viral infections, etc (6). CDRs of selected rodent antibodies are cut out and glued onto human antibody frameworks to minimize rejection by human patients.

Our predicted CDRs are slightly different from Chothia's. A careful comparison can be found from a hyperlink on our website to 'Andrew's Antibody Page' (http://www.biochem.ucl.ac.uk/~martin/abs/index.html).

Massive amounts of sequence data are being continuously published in the scientific literature. It is imperative to collect and properly align the sequences so that they can be used by as many researchers in this field as possible. We have previously published five editions of these sequences (see the Introduction of 2). In 1991, the fifth edition (2) consisted of three volumes. Currently, the database is more than five times as large. As of September 29, 1999, the Kabat database contained 1 599 375 and 2 517 756 nt for antibody light and heavy chain variable regions, respectively, as compared to 272 244 and 418 962 nt in 1991. Total numbers of entries, amino acids and bases of other categories of sequences can be obtained by using the 'Current Counts' hyperlink on our website. The collection is available on our website (http://www.immuno.bme.nwu.edu) which is free due to the generous support by various research grants from NIH since 1970.

Finally, numerous scientific papers have cited our database, quoting our fourth edition (7), fifth edition (2), or one of our more recent papers (8). On our part, we have been analyzing

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the Kabat Database during the past few years with reference to the total numbers of antibody and TCR V-genes, possible evolutionary selection processes, importance of antibody CDRH3s as related to their fine specificities, etc.

KABAT DATABASE

The Kabat Database may be accessed for searching, sequence retrieval and analysis by a few different methods: electronic mail, WWW and ftp. The electronic mail interface has been available since 1993, the WWW interface since 1995 and various formats of the database in electronic format for nearly a decade (8). Our data formats, searching tools, output formats and database structures have gradually been adopted by other immunological databases and interfaces.

Electronic mail interface

An electronic mail interface (seqhunt2@immuno.bme.nwu.edu) provides a non-interactive method for searching and sequence retrieval (9). Sending mail to the server address with the single word 'help' (no quotes) in the message body returns instructions for using the server.

All sequences classes are searchable and returnable. The query format allows making AND/OR/NOT constructed restrictions on the database and amino acid and nucleotide sequence pattern matching with allowable differences. Requests are processed as they are received and depending on the network traffic, take ~1–2 min to be searched and returned to the sender. The returned format is a fixed-line length record of 80 or fewer characters per line for ease in visual inspection and processing by user-written scripts or programs. The characters are plain text.

The query format for the sent request consists of two parts. The first part contains directives for the server to follow while the second part contains specifications of the search. Specification of the extent of data returned, the number of documents to return, starting document and whether plain ASCII text or PostScript should be used in the return format may be entered. Further, one can direct the server to return a distribution, the variability or unaligned raw data for the search specified.

The second part of the query contains the search restrictions on the database. Words separated by AND and OR may be used, as well as searching functions, like nucleotide/amino acid pattern matching and positional restriction matching.

There are basically three steps in translating and performing a search on the Kabat Database: generate the question or query, translate it into a format the server can recognize and decide on the output options desired of the returned matches. For example, if matches of mouse kappa light chains of anti-phosphorylcholine antibodies are desired, the query and restriction on the database would be:

Begin
@mouse and kappa and phosphorylcholine

The '@' before mouse tells the server that matches of the species mouse are desired, rather than searching through the entire database record for instances of the word 'mouse'. More complicated restrictions can be generated using parentheses for grouping and the minus sign '–' for NOT. Finding all rat and rabbit sequences which are not kappa light chains, and returning them as amino acid sequences in PostScript format would be constructed as:

PSAA
Begin
(rat and rabbit) and –kappa

Pattern matching is interpreted as the second part of an AND statement, such that finding all rat and rabbit sequences which are not kappa and contain the nucleotide pattern cagtacgtcag with three allowable mismatches, would be sent as:

Begin
(rat and rabbit) and –kappa [implicit AND]
#NM 3
cagtacgtcag

More examples of searching and output options may be found in the 'help' file returned from the server.

WWW interface

The WWW interface (8) to the Kabat Database: http://immuno.bme.nwu.edu contains searching and analysis tools as well as links to database download sites and other interesting databases. Most of the features found in the electronic mail interface are found in the WWW interface, as well as other tools. The WWW interface is more interactive than the Email and returns results faster, depending on the network traffic.

Searching and analysis tools

SeqhuntII. This grouping of programs allows searches through the annotations and sequence pattern matching of the amino acid and nucleotide sequence data with allowable mismatches. Like the Email server, restrictions on the database may be formulated as AND/OR/NOT constructs. Output extent, output format, maximum documents and starting document may be specified. Browsing of the output results in HTML format allows the user to view the database entries in an easy-to-read format. ASCII text may be selected as output for use in user-generated scripts and programs. PostScript generation allows for printing on a PostScript supporting printer. Sequence matching is returned aligned with the target sequence with nucleotide or amino acid differences from the database sequence displayed in a case change. Since the database contains only coding regions of genes and proteins, the query sequence should be a portion of the coding region being sought.

Variability. Variability and amino acid distributions of sequence groups may be generated for restrictions on the database. The variability plots are in PostScript format and may either be viewed on the screen with an appropriate PostScript viewer (e.g. GNU ghostscript or ghostview) or printed to a postscript-supporting printer. Plots for human and mouse TCR gamma and delta chain variable regions are shown in Figure 1. Scaling of the variability plots may be done allowing comparison of variability plots for different groupings of sequences. Distributions of the amino acids per position may be returned also, including the calculated variability for each position.

Sequence alignment. Alignment of user-entered coding regions of immunoglobulin light chains according to the Kabat numbering system can be performed. Because of the relatively few alignment options available for light chains, most sequences can be aligned. One can start with around 10 amino acid residues or 30 nt. There is no lower limit on the length of sequence to be matched. In some cases though, visual inspection and alignment is necessary, as is for heavy chain alignment,

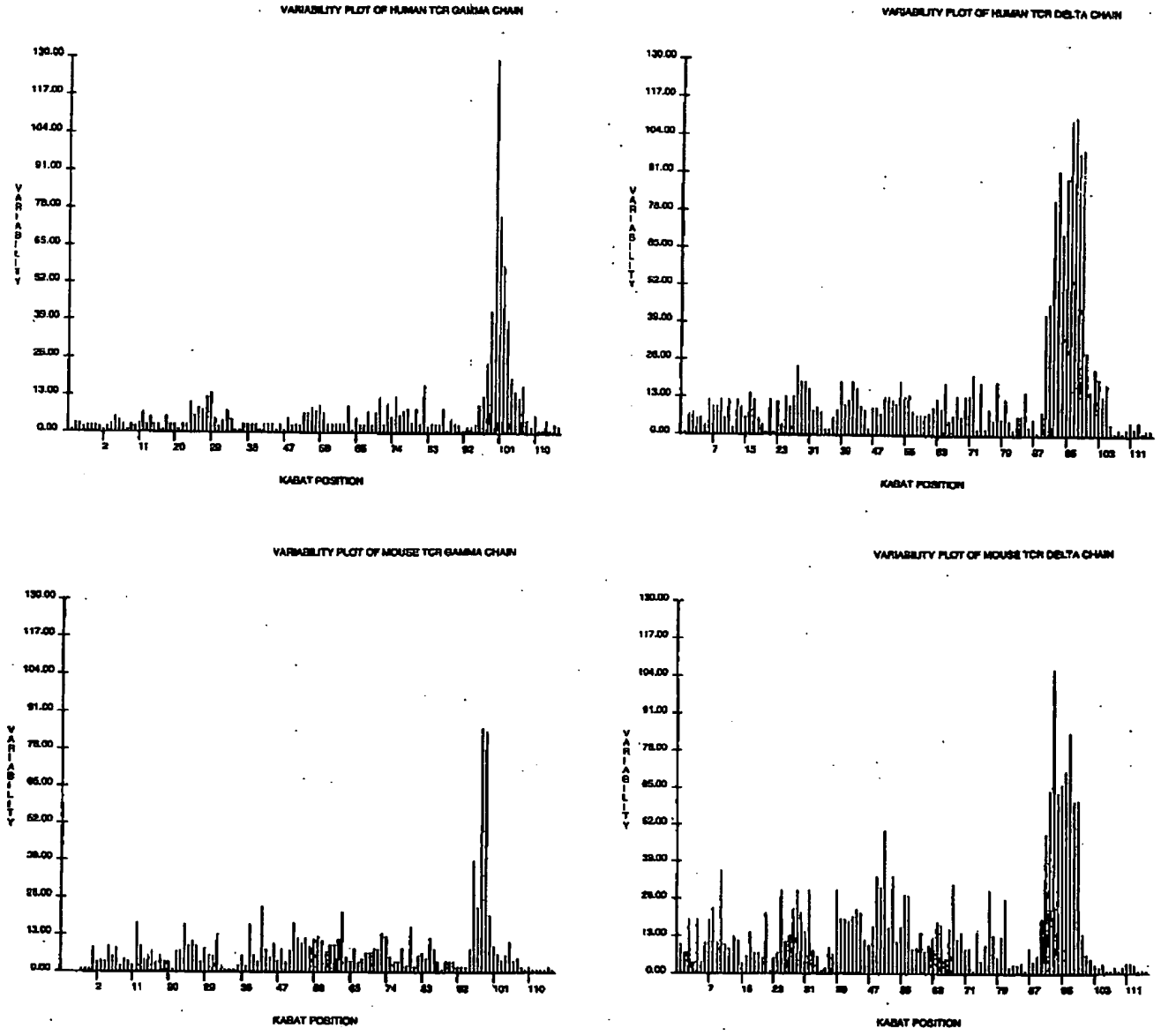

Figure 1. Variability plots for human and mouse TCR gamma and delta chain variable regions, using 377 human gamma, 1260 human delta, 297 mouse gamma and 461 mouse delta partial and complete sequences.

especially within the CDRH3 region, if additional codons or residues are inserted and denoted by '#'. If a suitable alignment counterpart from the database is not found for the target sequence, the user can contact us.

FTP. Various formats of the database are available for download from NCBI's repository under the directory 'kabat'. Currently active formats include a FASTA-like rawi sequence format and the database's fixed length format of 80 or fewer characters per line and vertical alignment. Four main variations on the fixed length format exist to properly visually display single translations, pseudogene translations, J-minigenes and D-minigenes. Other immunological databases have adopted similar formats as exemplified by the three letter code amino acid translation followed by single letter code. A 'dump' version of the database is periodically updated which contains unlimited line length records more suitable for mass processing on unix-based systems.

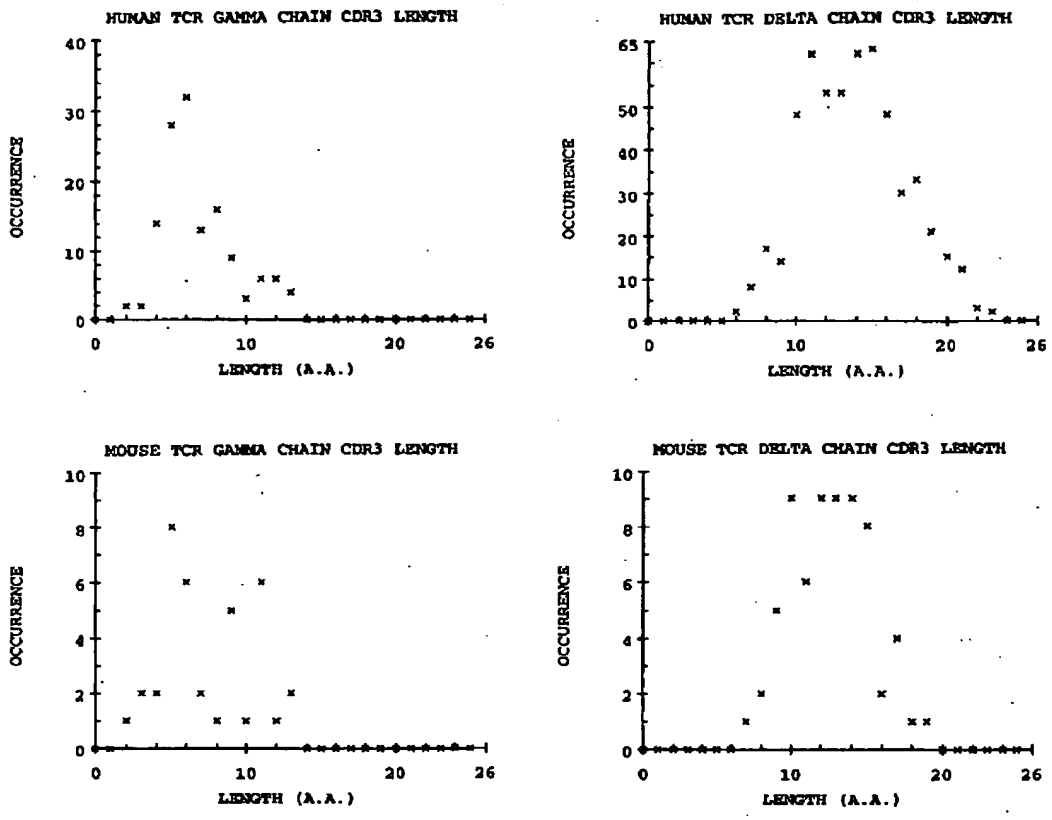

Figure 2. Length distributions of CDR3s of human and mouse TCR gamma and delta chains, based on 135 human gamma, 546 human delta, 37 mouse gamma and 66 mouse delta complete CDR3 sequences.

OTHER APPLICATIONS

As mentioned before, the Kabat Database was initially constructed for the purpose of identifying the antibody combining site (1). Starting from aligned amino acid sequences and using variability calculations, we have identified CDRs of antibody light and heavy chains, as well as those of TCRs. Such calculations can also provide useful predictions for MHC class I and II sequences (8), and to other aligned proteins sequences, e.g. HIV gp120, gp41, etc.

The importance of CDRH3 to confer fine specificity to antibodies was realized a few years ago (10). Furthermore, the unique CDRH3 nucleotide sequences have recently been used as a sensitive diagnostic test to detect residue B cell malignancies in cancer patients. Thus, many of these sequences have been determined. But most of them have been excluded from GenBank due to their relative short lengths. We have been meticulously collecting them, and realized the importance of their length distributions in antibodies of various specificities (11), and possible differences between CDRH3s of human and mouse (12). In the case of rabbit, the CDRH3s have less length variation than human and mouse. This may be compensated by the length variations of the CDRL3s (13).

The length variations of TCR alpha and beta chain CDR3s are very restricted (14). On the other hand, TCR gamma and delta chain CDR3s have more length variation, close to those of antibody heavy chains (Fig. 2). Whether they bind antigens directly is unclear.

During recent years, various research groups have decided to sequence the entire coding region of different antibody and TCR V-genes, in order to have an idea of their total numbers. On the other hand, we have discovered that pair-wise comparisons of V-gene nucleotide sequences in the Kabat Database provide very accurate estimations of their total numbers (15,16). In addition, such comparisons seem to suggest that antibody and TCR V-genes have evolved under different selective pressures (17). In the case of MHC class I sequences, comparison of their aligned sequences has elucidated a new mechanism of generating novel MHC class I molecules by random assortment of their a1 and a2 gene segments (18).

DISCUSSION

The Kabat Database has been around for 30 years. It has provided the immunology community a useful service, since it

not only is a sequence database but also incorporates vital aspects of the biology of the immune system. Various analytical methods have been developed to study the structure and function relations of proteins of immunological interest.
Electronic addresses:
http://immuno.bme.nwu.edu
seqhunt2@immuno.bme.nwu.edu
Citing the Kabat Database:
Authors using this database may cite this paper together with the electronic addresses.

ACKNOWLEDGEMENT

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REFERENCES

1. Wu,T.T. and Kabat,E.A. (1970) *J. Exp. Med.*, 132, 211–250.
2. Kabat,E.A., Wu,T.T., Perry,H., Gottesman,K. and Foeller,C. (1991) *Sequences of Proteins of Immunological Interest*, Fifth Edition. NIH Publication No. 91-3242.
3. Amit,A.G., Mariuzza,R.A., Phillips,S.E.V. and Poljak,R.J. (1986) *Science*, 233, 747–753.
4. Garcia,K.C., Degano,M., Stanfield,R.L., Brunmark,A., Jackson,M.R., Peterson,P.A., Teyton,L. and Wilson,I.A. (1996) *Science*, 274, 209–219.
5. Garboczi,D.H., Ghosh,P., Utz,U., Fan,Q.R., Biddison,W.E. and Wiley,D.C. (1996) *Nature*, 384, 134–141.
6. Jones,P.T., Dear,P.H., Foote,J., Neuberger,M.S. and Winter,G. (1986) *Nature*, 321, 522–525.
7. Kabat,E.A., Wu,T.T., Reid-Miller,M., Perry,H. and Gottesman,K. (1987) *Sequences of Proteins of Immunological Interest*, Fourth Edition. US Govt. Printing Off. No. 165-492.
8. Johnson,G., Kabat,E.A. and Wu,T.T. (1996) In Herzenberg,L.A., Weir,W.M., Herzenberg,L.A. and Blackwell,C. (eds), *Weir's Handbook of Experimental Immunology I. Immunochemistry and Molecular Immunology*, Fifth Edition. Blackwell Science Inc., Cambridge, MA, pp. 6.1–6.21.
9. Johnson,G., Wu,T.T. and Kabat,E.A. (1995) In Paul,S. (ed.), *Antibody Engineering Protocols*. Humana Press, pp.1–15.
10. Kabat,E.A. and Wu,T.T. (1991) *J. Immunol.*, 147, 1709–1719.
11. Johnson,G. and Wu,T.T. (1998) *Int. Immunol.*, 10, 1801–1805.
12. Wu,T.T., Johnson,G. and Kabat,E.A. (1993) *Proteins*, 16, 1–7.
13. Sehgal,D., Johnson,G., Wu,T.T. and Mage,R.G. (1999) *Immunogenetics*, 50, 31–42.
14. Johnson,G. and Wu,T.T. (1999) *Immunol. Cell Biol.*, 77, 391–394.
15. Johnson,G. and Wu,T.T. (1997) *Genetics*, 145, 777–786.
16. Johnson,G. and Wu,T.T. (1997) *Immunol. Cell Biol.*,75, 580–583.
17. Johnson,G. and Wu,T.T. (1997) *J. Mol. Evol.*, 44, 253–257.
18. Johnson,G. and Wu,T.T. (1998) *Genetics*, 149, 1063–1067.

(12) **United States Patent**
Hart et al.

(10) **Patent No.: US 6,875,433 B2**
(45) **Date of Patent: Apr. 5, 2005**

(54) **MONOCLONAL ANTIBODIES AND COMPLEMENTARITY-DETERMINING REGIONS BINDING TO EBOLA GLYCOPROTEIN**

(75) Inventors: **Mary Kate Hart**, Frederick, MD (US); **Julie Wilson**, Birmingham, AL (US)

(73) Assignee: **The United States of America as represented by the Secretary of the Army**, Washington, DC (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 82 days.

(21) Appl. No.: **10/226,795**

(22) Filed: **Aug. 23, 2002**

(65) **Prior Publication Data**

US 2004/0053865 A1 Mar. 18, 2004

(51) **Int. Cl.**[7] **A61K 39/42**

(52) **U.S. Cl.** 424/159.1; 424/130.1; 530/300; 435/345

(58) **Field of Search** 424/159.1, 130.1; 435/91.1, 69.1, 345; 530/300

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,792,462	A	8/1998	Johnston et al.	424/199.1
5,977,316	A *	11/1999	Chatterjee et al.	530/387.2
6,340,463	B1	1/2002	Mitchell et al.	424/263.1
6,630,144	B1 *	10/2003	Hart et al.	424/159.1
2003/0224015	A1	12/2003	Hart et al.	
2004/0146859	A1	7/2004	Hart et al.	

FOREIGN PATENT DOCUMENTS

WO	WO 96/37616	11/1996
WO	WO 99/32147	7/1999
WO	WO 00/00617 A2	1/2000
WO	WO 01/016183 A1 *	3/2001

OTHER PUBLICATIONS

Maruyama et al, Journal of Virology, Jul. 1999, vol. 73, No. 7, pp. 6024–6030.*

Wilson et al., "Vaccine Potential of Ebola Virus VP24, VP30, VP35 and VP40 Proteins", Virology 286, pp. 384–390 (2001).

Wilson and Hart, "Protection from Ebola Virus Mediated by Cytotoxic T Lymphocytes Specific for the Viral Nucleoprotein", Journal of Virology, Mar. 2001, vol. 75, No. 6, pp. 2660–2664.

Pushko et al., "Venezuelan Equine Encephalitis Virus Replicon Vector: Immunogenicity Studies with Ebola NP and GP Genes in Guinea Pigs", Vaccines 97, Molecular Approaches to the Control of Infectious Diseases, Cold Spring Harbor Laboratory Press, 1997, pp. 253–258.

Geisbert et al, "Evaluation in Nonhuman Primates of Vaccines Against Ebola Virus", Emerging Infectious Diseases, vol. 8, No. 5, May 2000, pp. 503–507.

Pushko et al., "Recombinant RNA Replicons Derived from Attenuated Venezuelan Equine Encephalitis Virus Protect Guinea Pigs and Mice from Ebola Hemorrhagic Fever Virus", JVAC, Vaccine, pp. 1–12.

Abstract, W33–5, Hooper et al., "DNA Vaccination Against Poxviruses Using Combinations ofIMV and EEV Immunogens", Jul. 2000, American Society for Virology Meeting.

Abstract, P23–6, Hooper et al., DNA Immunization with the Vaccinia L1R and/or A33R genes, Jul. 1998, Poster at American Society for Virology Meeting.

Meyer et al., "Identification of Binding Sites for Neutralizing Monoclonal Antibodies on the 14–kDa Fusion protein of Orthopox Viruses", Virology 200, pp. 778–783 (1994).

Czerny and Mahnel, "Structural and functional analysis of orthopoxvirus epitopes with neutralizing monoclonal antibodies", J. General Virology (1990), vol. 71, pp. 2341–2352.

Hooper et al., "DNA Vaccination with Vaccinia Virus L1R and A33R Genes Protects Mice Against a Lethal Poxvirus Challenge", Virology 266, pp. 329–339 (2000).

Vazquez and Esteban, "Identification of Functional Domains in the 14–Kilodalton Envelope Protein (A27L) of Vaccinia Virus", J. Virology, vol. 73, No. 11, Nov. 1999, pp. 9098–9109.

Vazquez et al., "The Vaccinia Virus 14–Kilodalton (A27L) Fusion Protein Forms a Triple Coiled–Coil Structure and Interacts with the 21–Kilodalton (A17L) Virus Membrane Protein through a C–Terminal alpha–Helix", J. Virology, vol. 72, No. 12, Dec. 1998), pp. 10126–10137.

Rodriguez et al., "The Vaccinia Virus 14–Kilodalton Fusion Protein Forms a Stable Complex with the Processed Protein Encoded bythe Vaccinia Virus A17L Gene", J. Virology, vol. 67, No. 6, Jun. 1993, pp. 3435–3440.

Lai et al., "The Purified 14–Kilodalton Envelope Protein of Vaccinia Virus Produced in *Escherichia coli* Induces Virus Immunity in Animals", J. Virology, vol. 65, No. 10, Oct. 1991, pp. 5631–5635.

Rodriguez and Esteban, "Mapping and Nucleotide Sequence ofthe Vaccinia Virus Gene That Encodes a 14–Kilodalton Fusion Proteins", J. Virology, Nov. 1987, vol. 61, No. 11, pp. 3550–3554.

Rodriguez et al., "Isolation and Characterization of Neutralizing Monoclonal Antibodies to Vaccinia Virus", J. Virology, Nov. 1985, vol. 56, No. 2, pp. 482–488.

(Continued)

Primary Examiner—Ali R. Salimi
(74) *Attorney, Agent, or Firm*—Elizabeth Arwine

(57) **ABSTRACT**

In this application are described Ebola GP monoclonal antibodies, epitopes recognized by these monoclonal antibodies, and the sequences of the variable regions of some of these antibodies. Also provided are mixtures of antibodies of the present invention, as well as methods of using individual antibodies or mixtures thereof for the detection, prevention, and/or therapeutical treatment of Ebola virus infections in vitro and in vivo.

6 Claims, 2 Drawing Sheets

Val (SEQ ID NO:9). The epitopes or peptides recognized by the antibodies of the present invention and conservative substitutions of these peptides which are still recognized by the antibody are an embodiment of the present invention. These peptides offer a convenient method for eluting GP bound to MAb 6D8, 13F6, or 12B5 on immunoaffinity columns. For example, when an antibody which recognizes the epitope for MAb 6D8, 13F6 or 12B5 is used in an immunoaffinity column to purify Ebola GP, the peptide recognized by the antibody can be added to the immunoaffinity column to elute the Ebola GP. Further truncation of these epitopes may be possible, as would be understood by someone having ordinary skill in this art having this disclosure in hand.

Epitope mapping studies described in this application defined five competition groups of MAbs. Antibodies which compete with the monoclonal antibodies of the present invention for binding to GP are considered to recognize the epitopes of the antibodies and are considered equivalent to the antibodies of the present invention. The MAbs 13C6 and 6D3 recognize conformational epitopes comprising discontinuous Ebola virus amino acids. Antibodies which compete with MAbs 13C6 and 6D3 for binding to Ebola GP are considered to recognize discontinuous epitopes and are considered equivalent to the antibodies of the present invention. Assays for determining whether or not an antibody competes with an antibody of the present invention are known to a person with ordinary skill in the art and are described below. Table 2 below defines functional criteria of each of the monoclonal antibodies identified in the Examples below.

TABLE 2

Epitopes Bound by Ebola GP MAbs.

Competition Group	Ebola Viruses with Epitope*	Ebola GPs with Epitope†	Epitope Sequence on Ebola GP‡	Amino Acids§
1	Z	GP1	ATQVEQHHRRTDNDSTA	401–417
2	Z	GP1	HNTPVYKLDISEATQVE	389–405
3	Z	GP1	GKLGLITNTTAGVAGLI	477–493
4	Z, IC, S	GP1, sGP	discontinuous	1–295
5	Z, IC	GP1, sGP	discontinuous	1–295

*Reactivities of MAbs with Ebola Zaire (Z, isolates from 1976 and 1995), Sudan (S), and Ivory Coast (IC) viruses in ELISA.
†Determined by western blot reactivity with Ebola Zaire 1995 virions or by immunoprecipitation (FIG. 2).
‡MAbs bound two consecutive peptide sequences immobilised on SPOTS membranes. Each peptide was 13 amino acids long and had a 9 amino acid overlap with the preceding and subsequent peptides. Sequences in bold indicate the 9 amino acid overlapping consensus sequence found on both peptides bound by the MAbs. Peptides containing the entire amino acid sequence shown also competed the binding of MAbs to Ebola virions in ELISA.
§Amino acid numbers based on the GP sequence from Genbank (accession number U23187, A. Sanchez, S. G. et al. (1996) Proc. Natl. Acad. Sci., USA 93, 3602).

By further mapping of the binding site of the monoclonal antibodies described in this disclosure other peptides useful as a vaccine or a therapeutic can be determined using known methodologies. Therefore, in another aspect, this invention relates to a method for identifying protective antigenic epitopes, which method comprises the steps of (i) reacting a monoclonal antibody described herein to different overlapping fragments encompassing the complete antigen, (ii) identifying a fragment to which the protective antibody binds, (iii) narrowing the region containing sites further by reacting the monoclonal with smaller overlapping fragments encompassing the region identified in (ii), and (iv) choosing peptides to which the antibody binds as possible antigenic epitopes. The peptides can then be assayed for their ability to protect an animal from disease, or to reduce the severity of disease. Peptides defining antigenic protective epitopes can be used in a vaccine as described below and in the Examples.

The epitopes or peptides on Ebola GP to which the monoclonal antibodies bind can constitute all or part of an active vaccine. An active vaccine or therapeutic candidate might comprise these peptide sequences and others. These may be delivered as synthetic peptides, or as fusion proteins, alone or co-administered with cytokines and/or adjuvants or carriers safe for human use, e.g. aluminum hydroxide, to increase immunogenicity. In addition, sequences such as ubiquitin can be added to increase antigen processing for more effective immune responses.

Antibody molecules produced in vivo comprise two identical heavy chains that are covalently bound and two identical light chains, each of which is covalently bound to a heavy chain. Heavy and light chains each have one variable region and three constant regions. Within the variable regions of light and heavy chains are hypervariable sequences called complementarity-determining regions flanked by framework regions. The binding specificity of an antibody is conferred by its combination of complementarity-determining regions. There are three complementarity-determining regions on the light chain and three on the heavy chain of an antibody molecule. Together, these form the 3-dimensional cavity that will bind (hold) an epitope on an antigen. Although these regions are hypervariable, a particular complementarity-determining region on one antibody may also be found on antibodies with different specificities, as it is the total combination of complementarity-determining regions that is important. Generally, binding specificity is determined by the complementarity-determining regions on both chains, although it has been suggested that the complementarity-

determining regions on the heavy chain do not contribute to specificity when the light chain is produced by a gene called lambda x. Identification of the complementarity-determining regions is useful for changing the "speciation" of an antibody, for example changing a mouse antibody to a humanized form suitable for human use, because one would want to preserve the complementarity-determining regions so as not to eliminate the binding specificity. Using the numbering system of Kabat et al, (NIH Publication No. 91-3242, 1991) in which the signal sequences of the heavy and light chains are indicated with negative numbers, the complementarity-determining regions of the light chain are between amino acids 24–34 (CDR1), 50–56 (CDR2) and 89–97 (95 a–f, CDR3). The complementarity-determining regions of the heavy chain are between amino acids 31–35 (35 a–b, CDR1), 50–65 (52 a–c, CDR2), and 95–102 (100 a–k, CDR3). Insertions of extra amino acids into the complementarity-determining regions can be observed and their locations are represented above in parentheses, e.g 95 a–f. Deletions are also observed, for example in CDR3 of some types of heavy chains.

Throughout this description we refer to the CDRs in terms of both the amino acid sequence and the location within the light or heavy chain. As someone having ordinary skill in this art would understand, the "location" of the CDRs is conserved between species, but through the use the well known Kabat system—an arbitrary numbering system that aligns sequences. Therefore, according to the Kabat system, the first invariant amino acid of a given type of light chain might be used to define the CDR beginning at, for example, "position 24" even if there are not 23 preceding amino acids. Therefore, for the purposes of the description of this invention we are defining CDRs as according to the Kabat system which is accepted in the art. The Kabat system aligns the Mab sequences of different species, for example mouse and human, such that all species have CDRs aligned at the same numbered "positions". Alignment of the sequences occurs through the identification of invariant residues in either the CDR or the framework regions adjacent to the CDR. There are different forms of light and heavy chain variable regions that differ in the use and location of the invariant residues, but Kabat et al. identify these. Using the nomenclature in the 1991 edition of Kabat et al, Mab EGP 6D8-1–2 uses a heavy chain variable region of the IIID type, and a kappa light chain type II. Mab EGP 13C6-1-1 uses a heavy chain variable region of the miscellaneous type, and a kappa light chain type V. Mab EGP 13F6-1-2 uses a heavy chain variable region of the IIID type, and a lambda x light chain.

The DNA sequence of the variable regions of the heavy chain of Mab EGP13C6-1-1 is represented in SEQ ID NO:11, and the amino acid sequence is represented in SEQ ID NO:12. For the heavy chain, the CDRs were identified as located at the following amino acid positions:

31–35b (where, as noted above, "b" signifies the insertion of an extra amino acid) having the amino acid sequence TSGVGVG with the last two amino acids representing insertions at 35a and 35b (SEQ ID NO:13),

50–65: having the amino acid sequence LIWWD-DDKYYNPSLKS (SEQ ID NO:14), and

95–102 (includes residues at 100c,h,j,k, where, as noted above, "c,h,j,k" signifies the insertion of extra amino acids): having the amino acid sequence RDPFGYDNAMGY where DNAM are 100 c,h,j,k, respectively (SEQ ID NO:15).

It is believed that all three of these CDRs are necessary for effective binding of the Mab EGP 13C6-1-1 to the epitopes of Ebola GP.

The DNA sequence of the variable regions of the light chain of Mab EGP 13C6-1-1 is represented in SEQ ID NO:16, and the amino acid sequence is represented in SEQ ID NO:17. For the light chain, the CDRs were identified as located at the following amino acid positions:

24–34: having the amino acid sequence—KASQNVGTAVA (SEQ ID NO:18)

50–56: having the amino acid sequence—SASNRYT (SEQ ID NO:19) and

89–97: having the amino acid sequence—QQYSSYPLT (SEQ ID NO:20).

It is believed that all three CDRs are necessary for effective binding of the Mab EGP13C6-1-1 to the epitopes of Ebola GP. The invention also contemplates monoclonal antibodies having sequences that are at least 90%, and preferably 95%, homologous to the heavy and/or light chain regions described here as SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:16 and SEQ ID NO:17, and which compete for binding Ebola GP. There can be a 5% variation normally in even the more conserved framework regions, and someone having ordinary skill in this art using known techniques would be able to determine without undue experimentation such homologous, competing monoclonal antibodies. The invention also contemplates monoclonal antibodies that compete with EGP13C6-1-1 for binding to Ebola GP, and which have the above-described CDRs in the appropriate positions as determined by the Kabat system in the light and/or heavy chains.

Specificity is conferred with both heavy and light chains, and not usually with just the heavy or light chain alone; therefore, it is preferred that when this monoclonal antibody is used to detect Ebola in a sample (as described below), or to prevent or treat Ebola infection (as described below), both heavy and light chains are present.

The DNA sequence of the variable regions of the heavy chain of Mab EGP6D8-1-2 is represented in SEQ ID NO:21, and the amino acid sequence is represented in SEQ ID NO:22. For the heavy chain, the CDRs were identified as located at the following amino acid positions:

31–35: having the amino acid sequence—RYWMS (SEQ ID NO:23)

50–65 (includes 52a): having the amino acid sequence—EINPDSSTINYTPSLKD (SEQ ID NO:24)

95–102 (has one deletion): having the amino acid sequence—QGYGYNY (SEQ ID NO:25)

It is believed that all three CDRs are necessary for effective binding of the Mab EGP6D8-1-2 to the epitopes of Ebola GP.

The DNA sequence of the variable regions of the light chain of Mab EGP 6D8-1-2 is represented in SEQ ID NO:26, and the amino acid sequence is represented in SEQ ID NO:27. For the light chain, the CDRs were identified as located at the following amino acid positions:

24–34, includes 27 a–e: having the amino acid sequence RSSQSIVHSNGNTYLE (SEQ ID NO:28)

50–56: having the amino acid sequence KASNRFS (SEQ ID NO:29) and

89–97: having the amino acid sequence LQGSHVPST (SEQ ID NO:30).

It is believed that all three CDRs are necessary for effective binding of the Mab EGP 6D8-1-2 to the epitopes of Ebola GP. The invention also contemplates monoclonal antibodies having sequences that are at least 90%, and preferably 95%, homologous to the heavy and/or light chain regions described here as SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:26 and SEQ ID NO:27, and which compete for binding Ebola GP. As noted above, there can be a 5%

1: Science. 1988 Mar 25;239(4847):1534-6. Related Articles, Links

Reshaping human antibodies: grafting an antilysozyme activity.

Verhoeyen M, Milstein C, Winter G.

Medical Research Council Laboratory of Molecular Biology, Cambridge, England.

The production of therapeutic human monoclonal antibodies by hybridoma technology has proved difficult, and this has prompted the "humanizing" of mouse monoclonal antibodies by recombinant DNA techniques. It was shown previously that the binding site for a small hapten could be grafted from the heavy-chain variable domain of a mouse antibody to that of a human myeloma protein by transplanting the hypervariable loops. It is now shown that a large binding site for a protein antigen (lysozyme) can also be transplanted from mouse to human heavy chain. The success of such constructions may be facilitated by an induced-fit mechanism.

PMID: 2451287 [PubMed - indexed for MEDLINE]

1: Nature. 1988 Mar 24;332(6162):323-7. Related Articles, Links

nature

Reshaping human antibodies for therapy.

Riechmann L, Clark M, Waldmann H, Winter G.

MRC Laboratory of Molecular Biology, Cambridge, UK.

A human IgG1 antibody has been reshaped for serotherapy in humans by introducing the six hypervariable regions from the heavy- and light-chain variable domains of a rat antibody directed against human lymphocytes. The reshaped human antibody is as effective as the rat antibody in complement and is more effective in cell-mediated lysis of human lymphocytes.

PMID: 3127726 [PubMed - indexed for MEDLINE]

RESEARCH

Improving antibody affinity by mimicking somatic hypermutation in vitro

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In vivo affinity maturation of antibodies involves mutation of hot spots in the DNA encoding the variable regions. We have used this information to develop a strategy to improve antibody affinity in vitro using phage display technology. In our experiment with the antimesothelin scFv, SS(scFv), we identified DNA sequences in the variable regions that are naturally prone to hypermutations, selected a few hot spots encoding nonconserved amino acids, and introduced random mutations to make libraries with a size requirement between 10^3 and 10^4 independent clones. Panning of the hot spot libraries yielded several mutants with a 15- to 55-fold increase in affinity compared with a single clone with a fourfold increased affinity from a library in which mutagenesis was done outside the hot spots. The strategy should be generally applicable for the rapid isolation of higher-affinity mutants of Fvs, Fabs, and other recombinant antibodies from antibody phage libraries that are small in size.

Keywords: phage display, single-chain antibody, hot spots, affinity improvement

Antibodies have been used with encouraging results in the treatment and diagnosis of cancer[1–6]. They can be used alone or as vehicles to deliver radionuclides and cytotoxic agents to cancer cells. However, the use of antibodies for the treatment and imaging of solid tumors is made difficult by their large size and slow tumor penetration[7,8]. This has led to the development of antibody fragments, especially scFvs, as substitutes for whole antibodies. Because of their small size, Fvs can penetrate rapidly into solid tumors[9] and have also been shown to have greater tumor specificity[10] compared with Fabs and IgGs. However, these advantages are offset by the loss of antigen binding affinity that occurs when IgGs are converted to scFvs[11]. Increasing affinity has been shown to improve selective tumor delivery of scFvs[12] and is likely to increase their usefulness in tumor imaging and treatment.

Improvements in affinity can be achieved in vitro by site-directed or random mutagenesis. Although antibody–antigen crystal structure can indicate which residues should be mutated to improve binding, atomic resolution structural data are not available for most antibodies. Therefore, several strategies based on phage display technology have been developed to select higher-affinity antibodies from libraries expressing mutated variants of Fabs and scFvs[13–22]. These approaches require the construction of huge and multiple libraries, which are often difficult to attain and may be expensive to produce.

We have sought to develop a strategy that would enable isolation of Fvs with increased affinity from a small library of variants. This approach is based on the fact that the DNA encoding the variable regions of antibodies contains mutational hot spots, which are nucleotide sequences where mutations are frequently concentrated during the in vivo affinity maturation process[23–25]. Several different types of variable-region hot spots, such as direct and inverted repeats, palindromes, secondary structures, and certain consensus sequences, have been proposed[25–30]. Among these, the consensus hot spot sequences have been studied in greatest detail. One of these consensus sequences is a tetranucleotide A/G-G-C/T-A/T (Pu-G-Py-A/T). The other type is represented by the serine codons AGY, where Y can be a C or a T[30,31].

The single chain Fv (scFv) chosen for this study, termed SS(scFv), was selected from a phage display library based on its recognition of the mesothelin antigen, which is expressed on the surface of ovarian cancers, mesotheliomas, and several other human cancers[32,33]. SS(scFv)-PE38 is an immunotoxin made by fusing SS(scFv) with a 38 kDa truncated mutant of *Pseudomonas* exotoxin A called PE38. When injected into mice carrying mesothelin-positive human tumors, SS(scFv)-PE38 causes regression of the tumors and is being developed for treatment of human cancers. The affinity of SS(scFv)-PE38 for mesothelin was found to be 11 nM (ref. 32). Improving the affinity of SS(scFv) is desirable to improve the cytotoxicity and antitumor activity of the immunotoxin. We report here on our studies in which we targeted random mutations to some of the selected hot spots in complementarity-determining region 3 (CDR3) of the light chain. Using this approach, we were able to isolate scFvs that had 15- to 55-fold increase in affinity from a small library of about 8,000 independent clones. Immunotoxins made with these high-affinity variants had more than a 10-fold increase in cytotoxic activity.

Results and discussion

Construction of the libraries. Of the 32 hot spots in SS(scFv), 14 are located in the variable heavy (VH) and 18 in the variable light (VL) chains. We chose the CDR3s to begin mutagenesis because they are the major sites of interaction with the antigen. We chose VL CDR3 over VH CDR3 because it undergoes fewer recombination events and is therefore closer to the germ line. In the CDR3 of VL there are two hot spots of the A/G-G-C/T-A/T type and one AGT serine codon. We began our experiments by concentrating on the tetranucleotide hot spots in VL CDR3. We randomized all the nucleotides of the codons that form whole or part of the tetranucleotide motif unless there was a special reason not to do so (discussed below).

The first phage library (Lib 89/93/94) involved randomizations of codons that are part of two tetranucleotide hot spots, one encoding residues 89 and 90 and the other residues 93 and 94 in the VL. Because Gln90 is highly conserved in subgroup VI (ref. 34), to which SS VL belongs, we chose to leave it unchanged and randomized residues 89, 93, and 94. A library in which three amino acids are randomized should have 8×10^3 different protein variants, and in terms of all different nucleotide combinations (using NNS oligonu-

cleotides where N=A,G,C, or T and S=G or C), the complete repertoire would contain 3×10^4 different clones. The library we made contained 6×10^4 clones and is expected to cover virtually all the possibilities.

After panning and analyzing Lib 89/93/94 we found that Gln89 was conserved in all the binders, but substitutions were tolerated at positions 93 and 94. Using this information, we constructed a second library, Lib 92-94, in which the AGT codon (another type of hot spot) for Ser92 was mutated along with the codons for Gly93 and Tyr94. The size of this library was 1×10^5.

As controls for our experiments we made two libraries (Lib 89-91 and Lib 95-97). Lib 89-91 was made to examine the effect of randomizing conserved residues within a hot spot and Lib 95-97 was made to study effects of randomizing residues outside the hot spots. In these libraries, three codons were randomized to allow comparison with the other two libraries, in which three codons located in rationally selected hot spots were randomized. The size of each of these libraries was also 1×10^5.

Panning of the libraries and enrichment of binders. The libraries were panned on immobilized recombinant mesothelin. The results of the panning experiments are shown in Table 1. We know that with an input of $2–6 \times 10^{11}$ phage the background is usually around $5–6 \times 10^4$. For Lib 89/93/94 and Lib 92-94 we obtained 2×10^5 phage from the first panning. This represents an enrichment of about threefold of phages that bind mesothelin. At the second round there was a large increase in enrichment, which was 150- and 130-fold, for the two libraries, respectively. The enrichment after the third round of panning for these libraries was about fivefold. The overall enrichment was about 2,000-fold. In contrast, there was no enrichment for the control libraries after the first round of panning, but thereafter there was a gradual increase in enrichment (10-fold after second panning and 200-fold after the third). The overall enrichment for both the control libraries was also about 2,000-fold. Although the overall enrichment for all the libraries was similar, the pattern of enrichment was strikingly different. The early and rapid enrichment of two libraries, Lib 89/93/94 and Lib 92-94, indicates that both contained a large number of binders. In contrast, the slow and gradual enrichment of the control libraries, Lib 89-91 and Lib 95-97, suggests that they contained fewer binders that were slowly enriched during successive rounds of panning.

Screening of clones for mesothelin binding. Next, we examined the binding properties of phage selected from each of the different libraries. For each library we screened 23 clones by ELISA from the second and third round of the panning. The results of this experiment are shown in Figure 1.

Panel 1 shows the mesothelin-binding activity of clones selected after panning of Lib 89/93/94. As evident from Figure 1, all 46 clones bound to mesothelin and could be grouped into four types: (1) 27 clones had very high ELISA signals (2.3–2.7); (2) 12 clones had high ELISA signals (1.75–2.2); (3) six clones had ELISA signals comparable to the parental SS(scFv) phage (shown by filled squares); and (4) one clone had an ELISA signal lower than the wild-type parental phage.

Panel 2 shows the mesothelin-binding activity of phage clones from the Lib 89-91. Of the 46 clones tested, 39 bound mesothelin. None had ELISA signal greater than the parental clone. Twenty-eight of these 39 clones had ELISA signals similar to the parental clone (1.25–1.5). The remaining 11 clones had ELISA signals lower than the parental type.

Panel 3 shows the mesothelin-binding activity of phage clones selected from Lib 92-94. Like those from Lib 89/93/94 all 46 clones tested bound mesothelin and could be categorized into the following four groups: (1) 31 gave very high ELISA signals (2.3–2.9); (2) eight clones had a high ELISA signal (1.8–2.2); (3) five clones gave ELISA signals similar to the parental type; and (4) two clones had ELISA signals lower than that of the parental type.

Panel 4 shows the mesothelin-binding activity of clones selected after panning of Lib 95-97. All 46 clones tested were found to bind mesothelin and could be arranged into three groups based on ELISA signals: (1) 22 gave ELISA signals slightly better than the parental type; (2) 20 clones had ELISA signals similar to the wild type; and (3) four clones had ELISA signals below the parental type.

Table 1. Enrichment of mesothelin binding phage from the libraries by panning on recombinant mesothelin.

Panning of	Panning round	Input number of phage	Recovered number of phage	Enrichment over previous round	Total enrichment
Lib 89/93/94	1	3×10^{11}	2×10^5	3*	
	2	3×10^{11}	3×10^7	150	
	3	10^{11}	5×10^7	5	2250
Lib 92-94	1	2.6×10^{11}	2.1×10^5	3*	
	2	2.8×10^{11}	3×10^7	132	
	3	2×10^{11}	9×10^7	5	1980
Lib 89-91	1	6.75×10^{11}	5.6×10^4	0	
	2	4.1×10^{11}	3.8×10^5	10	
	3	3×10^{11}	5.3×10^7	200	2000
Lib 95-97	1	2×10^{11}	6×10^4	0	
	2	2×10^{11}	8×10^6	10	
	3	10^{11}	7.9×10^7	196	1960

*Refers to enrichment over the background level of phage binding which is $5–6 \times 10^4$ for an input phage number of $2–6 \times 10^{11}$.

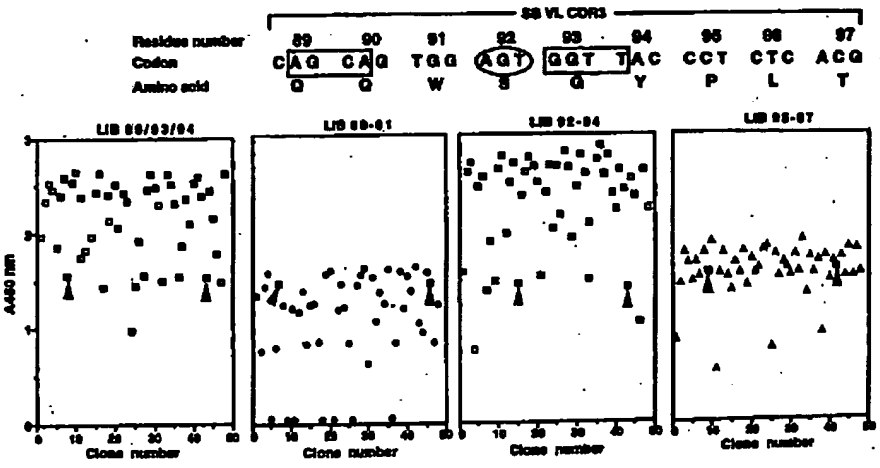


Figure 1. Mesothelin binding as judged by ELISA of phage clones selected after panning each of the four libraries. Each panel represents the ELISA results of clones from a library. Each symbol in a panel represents the ELISA signal of phage particles from a single clone. The filled squares in each panel (marked by arrows) represent the internal standard parental SS(scFv) phage. In the upper part of the figure the nucleotide and amino acid sequence of SS VL is indicated. The boxed region indicates the tetranucleotide hot spots and the oval circle indicates AGY type of hot spot.

Twelve phage preperations from each library were titrated and their numbers, as determined by colony forming units, were found to vary by less than twofold, indicating differences in ELISA signals were not due to differences in the number of input phage (data not shown). Additionally, no binding to bovine serum albumin was detected for phage particles from any of the clones (data not shown). These results indicate that: (1) the screening assay employed was dependable for identifying clones that made phage with better binding to mesothelin, and (2) targeting random mutations to rationally selected hot spots leads to isolation of better binders more easily than by random mutation of every codon of the CDRs.

Diversity of clones. Twenty-four ELISA-positive clones were sequenced from each library. The results are shown in Figure 2.

From Lib 89/93/94, four different sequences were obtained. All of the clones had preserved the parental residue, glutamine, at position 89. The residues at position 93 and 94 varied. The most abundant clone had G93K-Y94H mutations. The same clone also had the highest ELISA signal and was named SS1.

From Lib 89-91, all 24 clones sequenced had ELISA signals similar to wild-type SS(scFv), and all 24 had wild-type residues.

From Lib 92-94, seven different amino acid sequences were obtained (Fig. 2). The most abundant clones were D8 (S92G-G93F-Y94N) and C10 (S92G-G93S-Y94H). From this library we obtained two clones (E9 and E2) with identical amino acid sequence to SS1 and D7 from Lib 89/93/94. D8, C10, and SS1 are the clones with the highest ELISA signals.

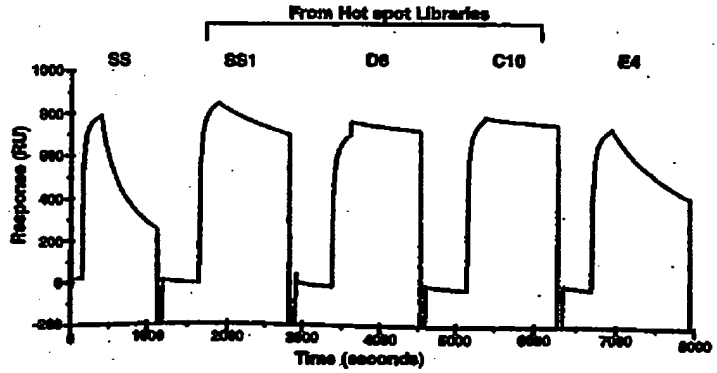
From Lib 95-97, 24 clones with ELISA signals similar to or greater than SS(scFv) were sequenced. Seventeen clones had the L96T mutation. The other seven clones had the wild-type amino acids. We call the L96T mutant E4(scFv).

In every case, the entire scFv coding region was sequenced to ensure that no other mutations had occurred. In terms of protein sequence, 10 different types of mutants were obtained. Many of these were encoded by several different nucleotide sequences. Nine of these 10 mutants were obtained from libraries in which mutations were targeted to rationally selected hot spots (Lib 89/93/94 and Lib 92-94). Thus, the presence of several clones with binding activity from these libraries agrees with the rapid enrichment pattern seen for these libraries. In contrast Lib 89-91, which has conserved residues in hot spots, failed to yield any binder other than the parental clone and Lib 95-97 in which mutations were targeted to non–hot spot regions yielded a single variant. The rarity of binders in these libraries also agree with their slow and gradual enrichment pattern.

Construction and purification of immunotoxins. Our laboratory is focused on the construction of immunotoxins for cancer therapy. In these molecules, the scFvs directed against a cancer cell surface antigen are fused in frame with a truncated mutant of *Pseudomonas* exotoxin A called PE38. The antigen-binding properties of the immunotoxins are determined by the scFv, and the PE38 performs an effector function that kills the cell to which the scFv binds. Therefore, by making and purifying immunotoxins containing the

Clone	Residue randomized	Clone name	VL CDR3 nucleotide and amino acide sequence								
			89	90	91	92	93	94	95	96	97
Parental	None	SS	cag	cag	tgg	agt	ggt	tac	cct	ctc	acg
			Q	Q	W	S	G	Y	P	L	T
Lib 89/93/94	89,93 and 94	SS1	Q				K	H			
		C5*	Q				G	H			
		D7*	Q				A	H			
		B7	Q				Q	I			
Lib 89–91	89–91	B4	Q	Q	W						
Lib 92–94	92–94	D8*				G	F	N			
		D9*				G	T	N			
		C10*				G	S	H			
		C3				G	D	F			
		C4*				G	D	H			
		E9*				S	K	H			
		E2				S	A	H			
Lib 95–97	95–97	E4*							P	T	T

Figure 2. Amino acid sequence of some of the ELISA-positive phage clones obtained after panning of the different libraries. Only sequences of regions that differ among the clones are shown. *Several clones with the same amino acid but different nucleotide sequences were found.



	SS	SS1	D8	C10	E4
K_{off} (s^{-1})	2.34×10^{-3}	2.22×10^{-4}	6.22×10^{-5}	6.56×10^{-5}	6.12×10^{-4}
K_{on} ($M^{-1}s^{-1}$)	2.13×10^{5}	3.11×10^{6}	2.06×10^{5}	3.08×10^{6}	2.11×10^{5}
K_D (nM)	11	0.72	0.3	0.2	3

Figure 3. Comparison of the BIAcore sensorgrams obtained for the binding of wild-type and mutated scFv immunotoxins to immobilized mesothelin. The values for k_{off}, k_{on}, and K_D are tabulated. RU, Resonance units. The *x*-axis represents time in seconds.

Table 2. Cytotoxicity of immunotoxins made with the parental and mutated scFvs on human ovarian adenocarcinoma cell line A1847 and T-cell leukemia line Hut102.

	IC_{50} (ng/ml) on		Relative increase in cytotoxicity	K_D	Relative increase in affinity
	A1847	Hut102			
SS	16	>1000	1	11	1
SS1	1.2	>1000	13	0.72	15
D8	1.4	>1000	11	0.3	37
C10	1.4	>1000	11	0.2	55
E4	8	>1000	2	3	4

For comparison, the K_D values obtained by BIAcore analysis and the relative increase in affinity of the mutants over the wild type immunotoxin are included.

mutant Fvs, we can determine the antigen-binding kinetics of the scFv and also study its cytotoxic activity in cell culture. Based on a more detailed ELISA with purified phage preparations of the different clones (data not shown), we selected four scFvs to construct and purify immunotoxins. Among these four, SS1, D8, and C10 represent the best clones obtained from libraries in which rationally selected hot spots were randomized, and E4 represents the only clone that had better binding than the parental SS(scFv) obtained from control libraries. The immunotoxins were purified to over 95% homogeneity and eluted as a monomer in TSK gel filtration chromatography (result not shown). These purified immunotoxins were used to determine the affinities of the scFvs and also to study their cell killing activities.

Binding characteristics of the immunotoxins. The binding characteristics of the scFvs selected by panning the phage libraries were determined using the corresponding purified immunotoxins for surface plasmon resonance studies. Figure 3 shows representative sensorgrams of the binding of immunotoxins to mesothelin and lists the values for the k_{on}, k_{off} and K_D. It is apparent that all the mutants (SS1, D8, C10, and E4) had better binding to mesothelin than the parental SS(scFv)-PE38. The on rates of SS1 and C10 were about 1.5-fold higher while those of D8 and E4 were about the same as the parental SS(scFv). The off rates of SS1, D8, C10, and E4 were decreased by 10-, 37-, 35-, and 3.8-fold, respectively, compared with the parental SS(scFv). Similar to what others have reported, the increased binding of the mutants observed in the present study was due mainly to a decrease in their off rates[18,20,21]. These data show that SS1, D8, and C10, which are derived from libraries in which rationally selected hot spots were mutated, showed a 15-, 37-, and 55-fold increase in affinity, respectively, over the parental Fv, whereas E4, which is the only mutant isolated from control libraries, showed a modest 3.7-fold increase in affinity over the parental SS(scFv).

Cytotoxic activity of the immunotoxins. For evaluating the usefulness of the higher-affinity scFvs as targeting agents in cancer therapy, we determined the cytotoxicity of the immunotoxins made with the different scFvs. Table 2 shows the cytotoxic activity of the various immunotoxins on antigen-positive A1847 and antigen-negative HUT 102 cell lines. On A1847 cells, SS1, D8, and C10 scFv-PE38 were over 10-fold more active than the wild-type SS(scFv) immunotoxin, while E4(scFv)-PE38 was only about twofold more active. Cytotoxicity on HUT 102 (antigen-negative cells) was not evident. Thus, the increase in affinity obtained by targeting mutations to rationally selected hot spots resulted in a large increase in cytotoxic activity. However, the increase in cytotoxicity was not proportionally related to the increase in affinity. For example, the IC_{50} of SS1(Fv)-PE38 and C10(Fv)-PE38 were very similar, whereas their affinities differed by about threefold. This difference could be due to the fact that the affinities were measured using recombinant protein made in *Escherichia coli*, whereas the cytotoxicity was measured on an ovarian cancer cell line expressing the antigen.

This strategy has been applied in our laboratory to another antibody that binds to a mutant form of epidermal growth factor receptor. It has been possible to isolate mutants with higher affinities in this study also (data not shown). These results strongly indicate that it should be easier to select better binding phage from small libraries made by targeting mutations to rationally selected hot spots than by the other currently used methods. In the current study, we had selected four positions for randomization, and three of them were found to be useful. The improvements obtained by our approach are comparable to those attained from the large phage libraries described in the literature[18–22]. We suggest that by mimicking the natural somatic hypermutation process, one can readily obtain recombinant antibodies with increased affinity for antigen either from immunized or nonimmunized libraries.

Experimental protocol

Construction of libraries. Phagemid pPSC 7-1 is a phage antibody display vector coding for the mesothelin-binding SS(scFv) (ref. 32). Analysis of the nucleotide sequence of SS(scFv) revealed 32 hot spots, of which three were selected for targeting mutations. These three are located in the CDR3 of the VL encoding residues 89, 90, 92, 93, and 94. Single-stranded uracil containing DNA of pPSC 7-1 was prepared as described[33]. A stop codon and a diagnostic *Hpa*I restriction site was introduced into the CDR3 of the VL by Kunkel's mutagenesis[34] to produce the phagemid pPSC 7-1-94. The stop codon was introduced to prevent overrepresentation of the wild-type sequence in the library as this mutagenesis approach gives a background of 10–12%. Uracil containing ssDNA of pPSC 7-1-94 was used as a template to construct two experimental and two control libraries using degenerate oligonucleotides. Oligonucleotide SS VL 89/93/94 5′-GCACCGAACGTGAGAGGSNNSNN-ACTCCACTGSNNGCAGTAATAAGTTGC-3′ was used for making library Lib 89/93/94. This oligonucleotide randomizes codons 89, 93, and 94 of the VL in SS(scFv) for all 20 different amino acids. As with all the other libraries described below, this oligonucleotide replaces the stop codon at position 94 with that of a tyrosine and does not create ochre and opal stop codons. The second library, Lib 92-94, was made using oligonucleotide SS VL Mut 92–94, 5′-GCACCGAACGTGAGAGGSNNSNNSNNCCACTGCTGGCAGTAATAAG-3′, which randomizes residues 92–94. Residues 92–94 are encoded by two different hot spots placed side by side. The third and fourth libraries were made as controls. The third library, Lib 89-91, was made with the oligonucleotide SS VL Mut 89–91, 5′-GCACCGAACGTGAGAGGGTAACCACT-SNNSNNSNNGCAGTAATAAGTTGC-3′, which randomizes residues 89–91. Residue 90 is a conserved residue in a hot spot. Residue 91 falls outside of the hot spot. The fourth library, Lib 95-97, was made with the oligonucleotide SS VL Mut 95–97 5′-CTTTGTCCCAGCACCGAASNNSNNSNNGTAAC-CACTCCACTGCTGCG-3′. It randomizes residues 95–97, all of which fall outside the hot spots.

Panning of the libraries and analysis of selected phage clones. Panning of the libraries was done as described[32]. For each library, 23 clones after the second round and 23 clones after the third round of panning were analyzed by ELISA for mesothelin binding as follows. Single colonies were inoculated into 125 µl of 2 × YT medium containing 100 µg/ml ampicillin and 2% glucose present in 96 wells of a microtiter plate. The plates were incubated at 37°C/200 r.p.m. in a humidified incubator for 3.5 h. Subsequently, 25 µl of culture from each well of these plates (master plates) were transferred to corresponding wells of another microtiter plate containing 125 µl of 2×YT medium with 100 µg/ml ampicillin, 2% glucose, and about 4×10^9 p.f.u./ml of M13KO7. This plate (phage plate) was then incubated for 2 h under the same conditions as described before. The phage plate was then centrifuged at 2,000 r.p.m. for 5 min at room temperature. The supernatant from each well was removed by gently shaking the plate in an inverted position. The cell pellet in each well was resuspended in 200 µl of 2×YT medium containing 100 µl/ml ampicillin and 50 µg/ml kanamycin. The plate was then incubated overnight as described before. After 14–16 h, the phage plate was cooled over ice and then centrifuged at 3,000 r.p.m. for 5 min at 4°C. The supernatant was used for phage ELISA and phage titration.

Construction of plasmids for expression and purification of immunotoxins. Phagemids pPSC 7-1-1, 7-1-2, 7-1-3, and 7-1-4 codes for SS(scFv) with the following mutations, respectively, in the VL: G93K-Y94H, S92G -G93P -Y94N, S92G-G93S-Y94H, and L96T. The scFvs from these phagemid vectors were PCR amplified using primer pairs New G2 *Nde*I and New G2 *Hind*III[32]. After purification, the PCR products were cloned into an *E. coli* expression vector as described[37]. The resulting plasmids, pPSC 7-2-1, pPSC 7-2-2, pPSC 7-2-3, and pPSC 7-2-4, respectively, had the scFvs fused in frame with a 38 kDa fragment of *Pseudomonas* exotoxin containing domains II and III. Recombinant proteins were produced from inclusion bodies as described before[37].

Surface plasmon resonance assay. The affinity of the scFvs was determined by studying the binding kinetics of the purified immunotoxins to mesothelin by the surface plasmon resonance (BIAcore) technique. In separate experiments, different amounts (200–1,000 resonance units) of recombinant mesothelin were immobilized onto a BIAcore sensor chip, CM5. Then each of the immunotoxins at a concentration of 380 nM was passed over the immobilized mesothelin. k_{on} was determined from a plot of (ln (dR/dt)/t) versus concentration, where R is response and t is time. k_{off} was calculated during the first 1–2 min of the dissociation phase of the sensorgrams. These association and dissociation rates were measured under a continuous flow rate of 10 µl/min. K_D was calculated by dividing k_{off} by k_{on}.

Cytotoxicity assays. Cytotoxic activity of the immunotoxins was determined as described[36] by inhibition of protein synthesis in mesothelin-positive human ovarian carcinoma line A1847 and mesothelin-negative T-cell leukemia line HUT 102. The IC_{50} is the amount of immunotoxin required to inhibit protein synthesis by 50%.

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1. Waldmann, T.A. et al. Radioimmunotherapy of interleukin-2Rα-expressing adult T-cell leukemia with yttrium-90-labeled anti-Tac. *Blood* 86, 4063–4075 (1995).
2. Pai, L.H., Wittes, R., Setser, A., Willingham, M.C., and Pastan, I. Treatment of advanced solid tumors with immunotoxin LMB-1: an antibody linked to Pseudomonas exotoxin. *Nat. Med.* 2, 350–353 (1996).
3. Maloney, D.G. et al. IDEC-C2B8 (rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma. *Blood* 90, 2188–2195 (1997).
4. Osterborg, A. et al. Phase II multicenter study of human CD52 antibody in previously treated chronic lymphocytic leukemia. *J. Clin. Oncol.* 15, 1567–1574 (1997).
5. Baselga, J., Norton, L., Albanell, J., Kim, Y.M. & Mendelsohn, J. Recombinant humanized anti-HER2 antibody (Herceptin (M)) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. *Cancer Res.* 58, 2825–2831 (1998).
6. Goldhirsch, A., Cortes, A.S., Castiglione-Gertsch, M. & Gelber, R.D. New treatments for breast cancer: breakthroughs for patient care or just steps in the right direction? *Ann. Oncol.* 9, 973–976 (1998).
7. Jain, R. Physiological barriers to delivery of monoclonal antibodies and other macromolecules in tumors. *Cancer Res.* 50, 814s–819s (1990).
8. Clauss, M.A. & Jain, R.K. Interstitial transport of rabbit and sheep antibodies in normal and neoplastic tissues. *Cancer Res.* 50, 3487–3492 (1990).
9. Yokota, T., Milenic, D.E., Whitlow, M. & Schlom, J. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res.* 52, 3402–3408 (1992).
10. Adams, G.P. et al. Highly specific *in vivo* tumor targeting by monovalent and divalent forms of 741F8 anti-C-erbB2 single chain Fv. *Cancer Res.* 53, 4026–4034 (1993).
11. Reiter, Y., Brinkmann, U., Lee, B. & Pastan, I. Engineering antibody Fv fragments for cancer detection and therapy: disulfide-stabilized Fv fragments. *Nat. Biotechnol.* 14, 1239–1245 (1996).
12. Adams, G.P. et al. Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. *Cancer Res.* 58, 485–490 (1998).
13. Gram, H. et al. *In vitro* selection and affinity maturation of antibodies from a naive combinatorial immunoglobulin library. *Proc. Natl. Acad. Sci. USA* 89, 3576–3580 (1992).
14. Low, N.M., Holliger, P. & Winter, G. Mimicking somatic hypermutation: affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. *J. Mol. Biol.* 260, 359–368 (1996).
15. de Kruif, J., Boel, E. & Logtenberg, T. Selection and application of human single chain Fv antibody fragments from a semi-synthetic phage antibody display library with designed CDR3 regions. *J. Mol. Biol.* 248, 97–105 (1995).
16. Griffith, A.D. et al. Human anti-self antibodies with high specificity from phage display libraries. *EMBO J.* 12, 725–734 (1993).
17. Barbas, C.F. et al. Molecular profile of an antibody response to HIV-1 as probed by combinatorial libraries. *J. Mol. Biol.* 230, 812–823 (1993).
18. Schier, R. et al. Isolation of high-affinity monomeric human anti-c-erbB-2 single chain Fv using affinity-driven selection. *J. Mol. Biol.* 255, 28–43 (1996).
19. Yelton, D.E. et al. Affinity maturation of the BR96 anti-carcinoma antibody by codon-based mutagenesis. *J. Immunol.* 155, 1994–2004 (1995).
20. Yang, W.-P. et al. CDR walking mutagenesis for the affinity maturation of a potent human anti-HIV-1 antibody into the picomolar range. *J. Mol. Biol.* 254, 392–403 (1995).
21. Pini, A. et al. Design and use of a phage display library. *J. Biol. Chem.* 273, 21769–21776 (1998).
22. Wu, H. et al. Stepwise in vitro affinity maturation of Vitaxin, an $\alpha_v\beta_3$-specific humanized mAb. *Proc. Natl. Acad. Sci. USA* 95, 6037–6042 (1998).
23. Neuberger, M. S. & Milstein, C. Somatic hypermutation. *Curr. Biol.* 7, 248–254 (1995).
24. Jolly, C.J. et al. The targeting of somatic hypermutation. *Semin. Immunol.* 8, 159–168 (1996).
25. Gonzalez-Fernandez, A., Gupta, S.K., Pannell, R., Neuberger, M.S. & Milstein, C. Somatic mutation of immunoglobulin λ chains: a segment of the major intron hypermutates as much as the complementarity-determining regions. *Proc. Natl. Acad. Sci. USA* 91, 12614–12618 (1994).
26. Golding, G.B., Gearhart, P.J. & Glickman, B.W. Patterns of somatic mutations in immunoglobulin variable genes. *Genetics* 116, 169–176 (1987).
27. Kolchanov, N.A., Solovyov, V.V. & Rogozin, I.B. Peculiarities of immunoglobulin gene structures as a basis for somatic mutation emergence. *FEBS Lett.* 214, 57–61 (1987).
28. Rogozin, I.B., Sredneva, N.E. & Kolchanov, N.A. Somatic hypermutagenesis in immunoglobulin genes. III. Somatic mutations in the chicken light chain locus. *Biochim. Biophys. Acta* 1089, 176–182 (1991).
29. Inlet, R.A. & Varade, W.S. Bias in somatic hypermutation of human V-H genes. *Int. Immunol.* 6, 1437–1443 (1994).
30. Wagner, S.D., Milstein, C. & Neuberger, M.S. Codon bias targets mutation. *Nature* 376, 732 (1995).
31. Goyenechea, B. & Milstein, C. Modifying the sequence of an immunoglobulin V-gene alters the resulting pattern of hypermutation. *Proc. Natl. Acad. Sci. USA* 93, 13979–13984 (1996).
32. Chowdhury, P.S., Viner, J., Beers, R. & Pastan, I. Isolation of a high-affinity stable single-chain Fv specific for mesothelin from DNA-immunized mice by phage display and construction of a recombinant immunotoxin with anti-tumor activity. *Proc. Natl. Acad. Sci. USA* 95, 669–674 (1998).
33. Chang, K. & Pastan, I. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. *Proc. Natl. Acad. Sci. USA* 93, 136–140 (1996).
34. Johnson, G., Kabat, E.A. & Wu, T.T. In *Weir's handbook of experimental immunology I. Immunochemistry and molecular immunology*, 6th edn Ch. 6 (eds Herzenberg, L.A. Weir, W.M., Herzenberg, L.A. & Blackwell, C.) 6.1–6.21 (Blackwell Science, Cambridge, Massachusetts; 1996).
35. Chowdhury, P.S., Vasmatzis, G., Beers, R., Lee, B. & Pastan, I. Improved stability and yield of a Fv-toxin fusion protein by computer design and protein engineering of the Fv. *J. Mol. Biol.* 291, 917–928 (1996).
36. Kunkel, T.A. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* 82, 488–492 (1985).
37. Brinkmann, U. Pai, L.H., FitzGerald, D.J. & Pastan, I. B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice. *Proc. Natl. Acad. Sci. USA* 88, 8616–8620 (1991).
38. Chaudhary, V.K. et al. A recombinant immunotoxin consisting of 2 antibody variable domains fused to Pseudomonas exotoxin. *Nature* 339, 394–397 (1989).